DEVELOPMENT OF ANTI-CANCER NANOFORMULATIONS BY

INCORPRORATION OF WATER-INSOLUBLE NATURAL BIOACTIVE

AGENTS INTO LIPID-BASED DRUG DELIVERY SYSTEMS

A THESIS SUBMITTED TO

THE GRADUATE SCHOOL OF

ENGINEERING AND NATURAL SCIENCES

OF ISTANBUL MEDIPOL UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF

MASTER OF SCIENCE

IN

BIOMEDICAL ENGINEERING AND BIOINFORMATICS

By

Reem Karroum

December, 2022

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By Reem Karroum

09 December 2022

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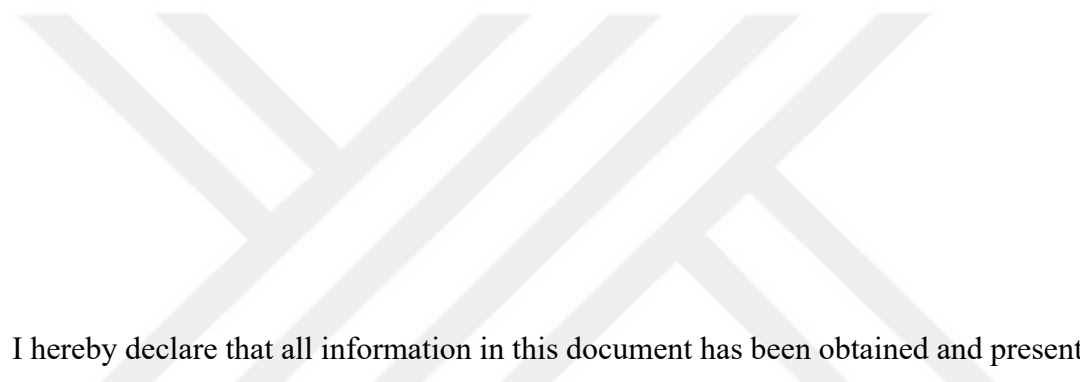
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ACKNOWLEDGEMENT

Reem Karroum

December, 2022

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

SFN   : Sulforaphane
SFN-Em   : Sulforaphane-Emulsome
HPLC   : High-Performance liquid chromatography
DNA   : Deoxyribonucleic Acid
NP   : Nanoparticle
DDS   : Drug-Delivery System
UV-vis   : Ultra-violet visible
MCF-7   : Michigan Cancer Foundation – 7
SEM   : Scanning Electron Microscopy
CLSM   : Confocal Laser Scanning Microscope
PDI   : Polydispersity Index
Bax   : Bcl2 associated X
Bcl2   : B-cell lymphoma 2
DMSO   : DimethylSulfoxide
DPPC   : Dipalmitoylphosphatidylcholine
NF-KB   : Nuclear factor kappa-light-chain-enhancer
PBS   : Phosphate-Buffered saline
TNFa   : Tumor necrosis factor alpha

DOĞAL BİYOAKTİF MADDELERİN LİPİD BAZLI İLAÇ TAŞIMA SİSTEMLERİNE EKLENMESİYLE ANTİ-KANSER NANOFORMÜLASYONLARININ GELİŞTİRİLMESİ

ÖZET

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Aralık, 2022

Brokoli dünya çapında tanınan ve çok tüketilen bir sebzedir, turpgiller kategorisine girer ve meme, cilt ve prostat kanserlerine karşı etkili olduğu kanıtlanmıştır. Bu anti-kanser özelliği, brokoli içindeki sülforafanın varlığından kaynaklanmaktadır.

Proje aşağıdaki hedeflere ulaşmayı amaçlamaktadır: Emülzomlarda enkapsülasyon yoluyla sülforafanın çözünürlüğünün, biyoyararlanımının ve stabilitesinin arttırılması; Ortalama boyutu yaklaşık 200 nm olan Sulforaphane-Emulsome (SFN-Em) formülasyonunun sentezi; MCF-7 kanser hücrelerine sülforafanın verilmesi; Sağlıklı hücreler (MCF-10A) üzerinde küçük bir etki ile MCF-7'ye SFN-Em verilmesi yoluyla anti-kanser etkisinin elde edilmesi. Kanser karşıtı etkinin kantitatif bir göstergesi olarak, hücre proliferasyonunun daha iyi veya eşit bir şekilde önlenmesinin başarılması, sülforafanın IC50 değerine göre değerlendirilecektir.

Deneyimize boş emülzomları sentezleyerek başladık, ardından sülforafan-emülzom formülasyonu izledik. Bu projenin ilk bölümü, örneklerin boyutunu (246 nm) ve potansiyelini (-22,5 mV) araştırmak için zetasizer, ardından tek tip küresel yapıyı göstermek için hem SEM hem de CLSM olan mikroskopi analizini içeren karakterizasyon tekniklerini içeriyordu. , ardından emülzomdaki sülforafan konsantrasyonunu (288 uM) elde etmek için HPLC kantifikasyon çalışmaları yapılır.

İkinci bölümün odak noktası hücre kültürü araştırmasıydı; MCF-7 hücre dizileri, hücre yaşayabilirliği üzerine araştırmalar için kullanılmadan önce üç gün süreyle çoğaldı. Daha sonra incelemeleri yürütmek için MTT testi kullanıldı.Sonuçlar daha sonra incelendi ve sırasıyla 24, 48 ve 72 saat geçtikten sonra %60, %57 ve %56 hücre canlılığı ortaya çıktı. Sulforaphane-emülzomun sağlıklı epitel hücreleri üzerinde herhangi bir zararlı etkisinin olmadığını doğrulamak için, aynı işlem 24, 48 ve 72 saat sonra %85, 82 ve 80 hücre canlılığı değerleri elde edilerek MCF10A sağlıklı hücre hatlarından oluşan kontrol grubuna da uygulandı. , sırasıyla.

Sülforafanın anti-kanser etkisini daha fazla doğrulamak için, 10uM sülforafan-emülzom uygulandığında erken apoptozun indüklendiğini (~%30) daha fazla kanıtlayan akış sitometrisi gerçekleştirilerek apoptoz çalışmaları gerçekleştirildi.

Elde edilen veriler, sülforafan yüklü emülzomların etkinliğini kanıtlamak için yeterliydi ve bu, gelecekteki çeşitli fikirler ve sülforafanın çeşitli özelliklerini araştırmak için yapılabilecek ileri çalışmalar için fikir verdi. Örneğin, genetik yolu ve farklı kanser türleri üzerindeki çeşitli etkileri test edilebilir.

Anahtar sözcükler: Sulforaphane, MCF7, kanser, ilaç salınımı

DEVELOPMENT OF ANTI-CANCER NANOFORMULATIONS BY INCORPORATION OF WATER-INSOLUBLE NATURAL BIOACTIVE AGENTS INTO LIPID-BASED DRUG DELIVERY SYSTEMS

ABSTRACT

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MSc in Biomedical Engineering and Bioinformatics

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December, 2022

Broccoli is a well-known and highly consumed vegetable around the world, it falls into the cruciferous category, which is proven to be effective against breast, skin, and prostate cancers. This anti-cancer property stems from the presence of sulforaphane within the broccoli.

In this study, we aim to investigate this cancer chemo-preventive feature by encapsulating sulforaphane within a lipid-based drug delivery system known as emulsomes. We started our experiment by synthesizing blank emulsomes, followed by sulforaphane-emulsomes formulation. The first part of this project contained the characterization techniques which included: zetasizer to investigate the size (246 nm) and the potential (-22.5 mV) of the samples, then microscopy analysis took place, both SEM and CLSM to show the uniformed spherical structure, followed by HPLC quantification studies to obtain the sulforaphane's concentration in the emulsome (288 µM).

The focus of the second section was on cell culture research; MCF-7 cell lines were proliferated for three days before being employed for investigations on cell viability. Later, the MTT test was used to conduct the investigations. The results were then examined revealing a 60, 57, and 56% cell viability after the passage of 24, 48, and 72 hours, respectively. To confirm that sulforaphane-emulsome has no harmful effects on healthy epithelial cells, the same process was then applied to the control group of MCF10A healthy cell lines, obtaining 85, 82, and 80% cell viability values after 24, 48, and 72 hours, respectively. To further affirm sulforaphane's anti-cancer effect, apoptosis studies were carried out by performing flow cytometry, which further proved the induction of early apoptosis (~30%) when 10uM of sulforaphane-emulsome was administrated.

The obtained data were sufficient to prove the efficacy of sulforaphane-loaded emulsomes, which provided insight for various future ideas and further studies that could be done to investigate the various properties of sulforaphane. For example, it can be tested for its genetic pathway and various effect on different cancer types.

Keywords: Sulforaphane, MCF7, cancer, drug delivery.

CHAPTER 1

1.   INTRODUCTION

On a worldwide scale, cancer is reported to be the prominent cause of death, covering approximately 10 million deaths in the year 2020. To put it in other terms, close to one in six deaths are cancer-related according to the world health organization (WHO). The most common cancer types are known to be breast, lung, colon, rectum, and prostate cancers [1].

Cancer, also referred to as malignant tumors and neoplasms, is a standard term indicating the quick formation of abnormal cells that develop beyond their common confines. This irregular progress results in them attacking neighboring organs and spreading to other parts of the body, which is identified as metastasis. Reports have presented that extensive metastasis are the major cause of death from cancer [2].

Noticeably, the prime risk aspect in most cancer cases is the simple fact one is getting older. More than three-quarters of all people suffering from cancer are usually 60 years old and over. That originates from the fact that cancer is a disease of genes, the DNA code that carries commands and directives for all the minuscule mechanisms inside one's cells. With the passage of time, faults gather in this code and these mistakes revitalize the cell's destination toward becoming carcinogenic [3]. Based on that, the longer one lives, the more he is subjected to the accumulation of errors within his genome. So, as time passes, the risk of cancer rises.

Drug candidates that are extracted from natural resources present a renewed attentiveness, especially when compared to present artificial drugs used in the pharmaceutical market due to their several drawbacks. These processed medications are characterized by their perilous side effects and their high costs due to the complex fabrication procedure. Moreover, they lack efficacy and often result in an increasing rate of drug resistance [4].

An isothiocyanate entitled Sulforaphane (SFN), is a natural molecule found in cruciferous vegetables, mostly broccoli sprouts, and is reported to have a wide-ranging protective effect on the body. On a molecular basis, Sulforaphane has a 4- (methyl sulfinyl) butyl group attached to the nitrogen and has the formula of $C_6H_{11}NOS_2$ [5].

SFN is obtained through the hydrolysis process of glucoraphanin, a compound mainly found in the young sprouts of broccoli and cauliflower. Glucoraphanin within the broccoli is converted to sulforaphane by an enzyme known as plant myrosinase. If the plant myrosinase is denatured by cooking, bacterial myrosinase in the human colon can also perform the conversion of glucoraphanin to sulforaphane [6].

According to recent studies, Sulforaphane possesses various positive health effects as an antioxidant, antimicrobial, anticancer, anti-inflammatory, anti-aging, neuroprotective, and anti-diabetic agent [7]. In the following research, the main focus will be on the molecule's intrinsic anti-cancer property by referring to the research and clinical trials that have been done recently. Accordingly, sulforaphane appeared to be most effective against breast and prostate cancer but has also been studied for its various effects on other cancer types, such as colon, leukemia, pancreatic, and melanoma [8]

Sulforaphane gained special attention due to its ability to simultaneously modulate multiple cellular targets involved in cancer development. It impacts the three main stages of tumor development. Throughout the transformation phase, SFN offers DNA protection by the modulation of carcinogen-metabolizing enzymes and blocking the action of mutagens [8]. During the proliferation and invasion stage, SFN maneuvers the hindering of the proliferation of cells (Phase 2). It triggers the initiation of programmed cell death (known as apoptosis) (Phase 3). Moreover, Sulforaphane favors the inhibition of neoangiogenesis; which refers to the progression of benign tumors into malignant tumors (Phase 2); thereby, it prohibits metastasis formation (Phase 3) [9]. Sulforaphane is characterized by its therapeutic property since it is able to prevent, delay, and reverse preneoplastic lesions [10]

Despite its beneficial properties against carcinogenesis by altering its epigenetic events in the cells, the usage of Sulforaphane for therapeutic treatment is limited mainly due to its poor bioavailability [11]. Additionally, its hydrophobicity, sensitivity to oxygen, heat, alkaline conditions, and water instability restrict its usage in pharmaceutical industries [12].

Nanomedicine holds great potential for the delivery of lipophilic compounds such as Sulforaphane with the aid of the targeting moieties on their surface and the capability to increase the material's bioavailability through blood circulation. New nano-sulforaphane formulations are still getting strategized by researchers due to their positive outcomes in previous and ongoing trials since they offer enhanced solubility, stability, and cellular uptake efficacy.

In the following research, we will investigate the therapeutic potential of Sulforaphane-loaded Emulsomes against the MCF-7 breast cancer cell model. The project aims to achieve at following targets: Increase in solubility, bioavailability, and stability of the sulforaphane through encapsulation in Emulsomes; Synthesis of Sulforaphane-Emulsome (SFN-Em) formulation with an average size of around 200 nm; Delivery of sulforaphane to MCF-7 cancer cells; Achievement of anti-cancer effect via delivery of SFN-Em to MCF-7 with minor effect on healthy cells (MCF-10A). As a quantitative indication of the anti-cancer effect, achievement of a better or equal inhibition of cell proliferation will be evaluated based on the IC50 value of sulforaphane [13].

CHAPTER 2

2. THEORETICAL PART

2.1. Discovery of New Drug Candidates and Their Challenges in Medicine

The recent development perceived in combinative chemistry and drug design have guided the enhancements of drug candidates that are characterized by their elevated molecular weight, high level of lipophilicity, and low water solubility. One of the main challenges in this field is the pursuit of pioneering medicines that could assist in the management of diseases without comprising a negative effect on one's safety and altering the drug's effectiveness [14].

Despite the substantial achievement in new drug discovery, there are many medical conditions that still require effective therapy. Due to the excessive requests made to the market, and the rising rivalry between pharmaceutical companies to satisfy the high necessity, various corporations have rushed up the development procedure of drug discovery. As a result, a critical number of drugs got approved even though they lack many bio-applicable properties. It has been reported that around 40% of those drugs are comprised of poorly soluble molecules which thereby hinders their translation into clinical applications [15].

In addition to that, it has been exhibited that these advertised water-insoluble drugs have very low absorbency levels, limited bioavailability, a rapid metabolism process in which they are quickly eradicated outside of the body, accompanied by a poor safety profile and admissibility [16].

In order to accomplish a clinically appropriate efficacy and safety profile for drug discovery and development, various properties such as solubility and permeability should be concurrently optimized. With respect to the Biopharmaceutical Classification System (BCS), drugs can be sorted into four sections in regard to their solubility and permeability

properties. Class I comprises the high solubility and permeability drugs. Class II consists of low-solubility but high-permeability drugs. Class III is referred to the high solubility but low permeability drugs. Whereas Class IV drugs are the ones characterized by both low solubility and permeability [17].

Drugs such as phenytoin, glibenclamide, carbamazepine, and ibuprofen belong to BCS class II in which they have low solubility but a practical membrane permeability. The main method used to enhance the delivery of these drugs is by following the formulation strategies such as crystal modification, micronization, complexation, etc. They help in the amplification of the drug dissolution rate and aid in attaining prolonged solubilization [18].

However, drugs belonging to BCS class IV such as amphotericin B, furosemide, Docetaxel, Paclitaxel, Azathioprine, and Colistin are water-insoluble and characterized by a feeble membrane permeability (**Table 2.1**).

Table 2.1: List of BCS Class IV drugs, their usages, limitations and success rates [19].

Novel Drug	Category & uses	Success Rate	Limitations
Amphotericin B (AmB)	**Anti-fungal:** Serious fungal infections and leishmaniasis	51% [20]	- Irregular and reduced absorption - Subdued and inconstant bioavailability - Elevated dosage and numerous administration - Slim immersion interface - Inter and intra substance irregularity
Furosemide	**Diuretic:** It treats edema (i.e., fluid retention)	76% [21]	
Docetaxel (DTX)	Anti-cancer	25 – 40% [22]	
Paclitaxel (PTX)		43 – 50 %[23]	
Azathioprine	Immunosuppressant	45 – 58 %[24]	
Colistin	Anti-microbial	61.2%[25]	

The already mentioned techniques, those used to advance the absorption of class II are limited for class IV due to the permeability drawback. Thereby, the preferred resolution to develop the drug's bioavailability is to return to the initial synthetic phase and alter the

main structure in order to attain a suitable physiochemical profile. However, that process would be costly and time-consuming. Thus, an appropriate initial design is crucial in order to ascertain an effective product for the class IV BCS [19].

In order to defy the hurdles these drugs face while trying to attain a successful delivery, numerous approaches can be retained to refine the drugs' bioavailability and effective delivery. These approaches can be identified as polymeric nanoparticulate systems, crystal engineering, lipid-based delivery systems, self-emulsifying solid dispersions, and P-efflux inhibition strategies [26].

2.2.   Natural Active Biomolecules

Since the dawn of medicine, nature has offered us an enormous collection of drugs in order to control several diseases, in which chemical ingredients have been extracted from animals, plants and microbes and are used to treat diseases. Natural medicine took the spotlight by becoming a fragment of our everyday nutrition such as: turmeric, cardamom, garlic, onion, ginger, tulsi, cloves, etc. The exploration of biological yields as source of medicinal therapeutics reached its uttermost greatness back in the 1980's when morphine, the active alkaloid that was extracted from opium poppy plant was discovered [27].

Drug candidates that are extracted from natural resources present a renewed attentiveness, especially when compared to present artificial drugs used in the pharmaceutical market due to their several drawbacks. These processed medications are characterized by their perilous side effects and their high costs due to the complex fabrication procedure. Moreover, they lack efficacy and often result in an increasing rate of drug resistance [4].

Natural compounds represent a vital source for new drugs and function as a prototype for the synthetization of new drugs ranging from antibiotics to anti-cancer treatments. The World Health Organization (WHO) encourages the practice of natural herbal remedies in order to lower the usage of current synthesized conventional treatment methods. Lately, the spotlight is being shed on research studying bioactive natural compounds, identifying their chemical configuration, and investigating the medicinal potential of numerous plants and how they are capable of yielding composites that acquire lower toxicity levels than currently used molecules. Based on these advantages, natural compounds excelled in the field and started to gain a long-lasting reputation in pharmaceutical studies that treats several diseases including microbial diseases, inflammatory disorders and cancer [28].

A wide range of examples of these natural biomolecules are already in clinical use and the others getting tested in clinical trials, such as Vincristine, irinotecan, etoposide and paclitaxel, which are the most common compounds originating from plants. Whereas actinomycin D, mitomycin C, bleomycin, doxorubicin and l-asparaginase are drugs emerging from microbial sources [29].

One example of these natural molecules is organosulfur compounds that have been considerably studied due to their significant role in fighting cardiovascular diseases. The organosulfur family exhibited various biological effects that made it successfully applicable in this field, such as: antioxidant properties, anti-inflammatory effects, blockage of platelet accumulation, blood pressure and cholesterol levels reduction [30]. Garlic and onion mark the main source for the organosulfur currently being reviewed for cardiovascular protection. Recent data proposes that organ sulfides are capable of inhibiting ROS production, escalating the antioxidant status, enhancing the bioavailability of nitric oxide, and it aids in the hindrance of vascular inflammation [31]

Another example is Curcumin (diferuloylmethane), in which various studies proved the extensive range of biological and pharmacological properties exhibited by this compound. It was demonstrated that curcumin is active and functional against numerous significant human pathogens, such as: Staphylococcus, Streptococcus, and Enterococcus [32]. In addition to that, many studies were organized in order to estimate the antibacterial and antioxidant activity of essential oils extracted from specific spices and herbs, such as: bay leaf, black pepper, coriander, garlic, ginger, black mustered, onion and turmeric. The results displayed a promising antibacterial property, especially for coriander oil, cumin oil and mustard oil [33].

Moreover, favorable and selective anti-cancer properties have been perceived from Saffron (stigmata of Crocus sativus L.) both in vitro and in vivo, but not yet in clinic trials [34]. Additionally, data proved that the usage of the oil extracted from Punica granatum exhibited positive anticancer properties, in which it interfered with the proliferative activity, cell cycle and apoptosis [35]. Furthermore, extracts (vinca alkaloids) from the periwinkle plant Catharanthus roseus, also known as Vinca rosea were demonstrated to acquire several remedial effects, mainly famous for its anti-cancer activity. One of the most famous alkaloids isolated from this plant is vinflunine. Due to its promise results in vivo and in vitro, in which it exhibited microtubule dynamics

disruption, antiangiogenesis and prolonged multidrug resistance development, the compound is currently being tested in the last clinical trial phase [36].

Furthermore, an example of functional foods, their sources and potential benefits is shown in (**Table 2.2**). A functional food is similar to standard food used in everyday diet, but it differs in respect to its physiological advantages, its nourishment of bioactive molecules, and its ability to minimize the risk of enduring various chronic diseases [29].

Table 2.2: Potential benefits of food supplementation with functional components from natural sources.

Operative constituents	Origin	Prospective Advantages
Lycopene	Tomato Produces	Decreases the hazard of prostate cancer
Insoluble fibre	Wheat Fiber	Diminishes the danger of breast or colon cancer
Beta-glucan	Barleys	Guarding against heart illnesses and some cancers
Soluble fibre	Fleawort	Guarding against heart illnesses and some cancers
Conjugated linoleic acid (CLA)	Cheese, meat commodities	Develops body configuration, declines danger of specific cancers
Anthocyanidins	Fruits	Deactivate free militants, decrease hazard of cancer
Catechins	Tea	Deactivate uninhibited militants, decrease hazard of cancer
Flavonones	Citrus fruit	Deactivate uninhibited militants, decrease hazard of cancer
Lignans	Flax, rye	Hindrance of cancer, renal malfunction

On the other hand, the isothiocyanates, mainly sulforaphane have emerged to the spotlight with a booming anti-cancerous property. Bestowing to modern studies, Sulforaphane acquires various progressive health outcomes in different areas, in which it can operate against cancer, diabetes, microbes, inflammations, and aging. It can also be defined as an

antioxidant [7]. In the following research, the main focus will be on the molecule's intrinsic anti-cancer property by referring to the research and clinical trials that have been done recently. Many types of cancer were tested for the effect of SFN, such as colon, pancreatic, and leukemia, but it was demonstrated that this compound is frequently efficient in breast and prostate cancer [37].

2.3. Application of the Use of Nanotechnology in the Design of Novel Nanomedicine Approaches

The chemical composition and complexity of the abstracts isolated from natural resources are essential to determine the success rate of a formulation, since the compound is required to release its active ingredient at the appropriate position and at a calculated rate. Subsequently, a mean of transportation, also referred to as a vehicle, must simultaneously enhance the drug's solubility, diminish the degradation activity, and minimize toxicity all while adjusting the functional absorption and biological reaction [38].

The configuration and biological behaviors of respective therapeutic plant products are recognized and addressed by phytochemical and phytopharmacological. Most of the biologically active parts of extracts isolated exhibit a diminished absorption level, because they are incapable to bypass the lipid membranes, and are characterized with an elevated molecular size. Thereby, they result in a loss of bioavailability and efficiency. Flavonoids, tannins, and terpenoids fall into this category. Several studies have reported some natural herbal medicines that demonstrated a decent activity when tested in vitro, however that wasn't consistent when examined in vivo. Based on this, a variety of compounds are seldom used due to the fact they are conflicting with other constituents within the formulation or expose unfavorable properties [39].

Numerous nanotechnological approaches have been utilized to overcome these hurdles, such as: polymeric nanoparticles, solid lipid nanoparticles, liquid crystal systems, precursors systems for liquid crystals, liposomes, and microemulsions. The main function of these carriers is to permit substances with diverse properties to be synthesized within the same formulation. Also, they are capable of altering the compound's physiochemical property and its corresponding behavior in a biological setting. Nanotechnology is a revolutionary step that will shape the future of drug delivery systems and will improve the efficacy of the active composite constituting the synthesized nano-formulation. This approach is gaining more fame in the pharmaceutical market because these drug delivery

systems are capable of enhancing selectivity and effectiveness, guarding the compound against thermal or photodegradation, it aids in minimizing the side effects and monitoring the release of the active part [40]. Therefore, most of the studies focuses on the usage of nanoparticles to treat diseases such as HIV, diabetes, cancer and a vehicle for drug delivery.

Additionally, nanostructures help improve the solubility and stability, they are capable of effectively associate active substances with different hydrophilicity-lipophilicity degree.

Thereby, this technology can be utilized to target the delivery of a substance toward assigned tissues or organs [41]. Nanotechnology is gaining higher significance in medicine mainly due to its small size (100-10,000 times smaller than the human cell) and notorious targeted effects.

Due to the large surface area to small size ratio, the nanoscale vehicles can effortlessly interact with enzymes and receptors found on both the surface and the interwall of the cell. Through access acquisition to several regions of the body, the nanostructure is capable of identify the disease and deliver the treatment even at a micro level (**Figure 2.1).**
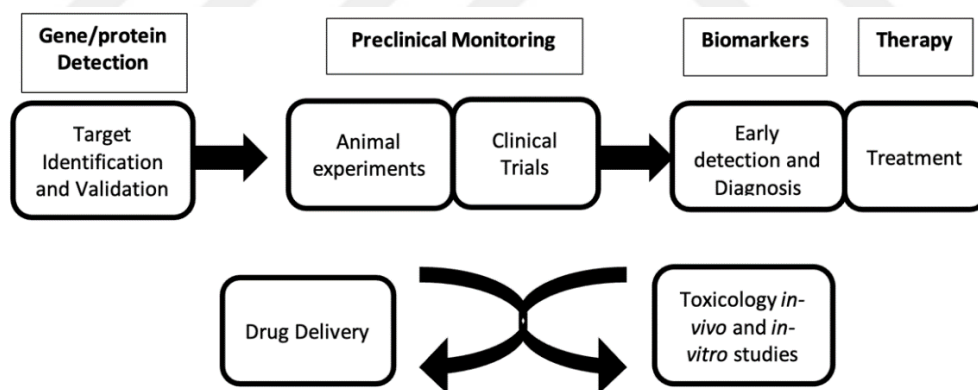
Figure 2.1: Nanotechnology plays a role in many stages of drug discovery and development [41].

Recently, the most favorable application of nanotechnology is drug delivery in order to overcome the main problem faced by the chemical entities which is their poor solubility. Nanotechnology have shed the light on targeted drug delivery and controlled drug release. These delivery systems are capable of delivering the drugs more effectively, increase the patient's fulfilment and subsequently, increase the product life cycle and the success rate (**Figure 2.2**). With respect to Dubin [42], drugs incline to execute a more effective profile and exhibit lesser side effects when synthesized within a nanoparticle. In addition, there

exist receptors of a nano-size over the cell's surface that is capable of distinguishing the drug and provoking an applicable response through providing and discharging the treatment at the time needed.
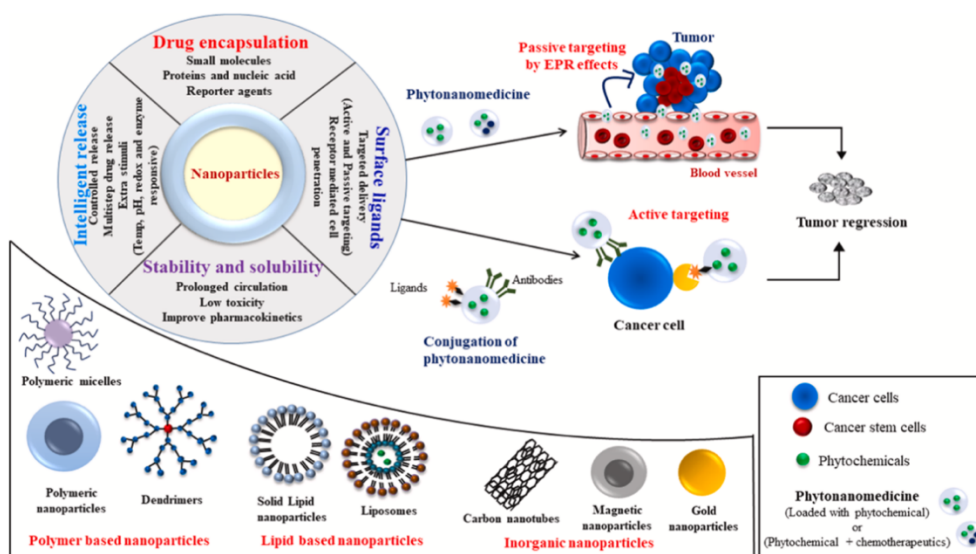
Figure 2.2: Illustration of adaptable drug delivery systems for anticancer phytochemicals and Phyto nanomedicine targeting techniques for full tumor regression[42] .

Hence, drugs can be loaded into the nanostructure trough three different methods: via encapsulation, surface attachment or entrapping. The attachment technique can be decided based on several factors, such as architecture and material of the nanostructure used, the drug type and the targeted location physiochemical properties [42]. Several alterations of these transporters are frequently used in order to regulate their properties in a appropriate manner. For instance, in order to enhance the stability and longevity of the drug delivery system, a specific targeting effects should be attained in order to recognize the organ or the tissue. Additionally, the sensitivity toward a specific stimulus within the pathological area should be investigated. Also, it is preferred to be supplied with visual data in regard of the carrier and its distribution inside the body [43].

A great number of publications concerned with drug delivery systems (DDS) clearly express several useful functions through the combination of different properties. Preferably, the DDS could be executed in a way which can instantaneously display the following four sets of properties: (1) High blood circulation duration; (2) Specific tangibility; (3) react to local stimuli features of targeted site, such as pH values, temperature, or outwardly applied heat, magnetic field, or ultrasound, which aids in the

discharge of the drug or alter some of its properties; (4) improved intracellular transfer of drugs and genes as needed [44].

2.4.   Sulforaphane; Origin, Medical History, Properties and Limitations

Broccoli is a vegetable linked to the Brassica oleracea group and it was believed to have been cultivated in the 1500s, it was transported to the UK in the beginning of the 1700s, and to the USA in the late 1700s, but wasn't widely known and consumed till the early 1920s[45]. During that century, the analysis of broccoli's diverse effects on health was at its primal. It started with Graham's study that demonstrated a dose-dependent association between the ingestion of broccoli (cruciferous vegetables in general) and protection against colon cancer [46]. After that, there have been a remarkable decrease in the risk of bladder cancer [47] and prostate cancer [48] associated with the consumption of cruciferous vegetables and broccoli.

Isothiocyanates (ITC) are one of the natural chemo-preventive families, they are formed through the hydrolysis process of their predecessor compound known as glucosinolates. Based on the cultivation method and genotype, the glucosinolates' amount within the Cruciferae family can significantly vary [49]. Within the diverse varieties of cruciferous vegetables, there are around 120 glucosinolates, each of them generating different aglycone metabolic yields including isothiocyanates [50]. Glucosinolates' general structure is comprised of β-D-thioglucose group, a sulfonated oxime group, and a variable side chain.

SFN and I3C are two significant and well-researched composites that are procured from cruciferous materials [51]. The glucosinolates that is progenitor to SFN is known as glucoraphanin (**Figure 2.3**). It is mainly found in broccoli, cauliflower, cabbage, kale and is abundantly expressed with a premier concentration in broccoli and broccoli sprouts [49]. Myrosinase enzymes are responsible for the hydrolyzation process of glucoraphanin into SFN. These enzymes are either released by the plant through the utilization course or found in our stomach.
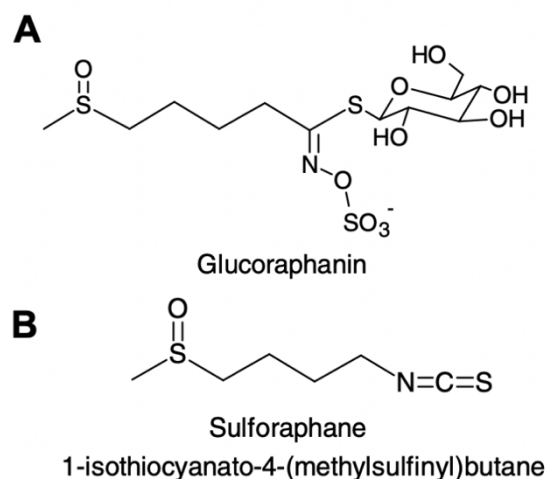
Figure 2.3: Structure of glucosinolates (a) glucoraphanin and its isothiocyanate hydrolysis yield (b) sulforaphane.

Sulforaphane is a nutritional compound characterized with its minimal toxic level. It is frequently and extensively utilized with cruciferous vegetables its administration to humans is typically well-accepted. Isothiocyanate SFN has been comprehensively reviewed in the former years for its defensive effect against a variety of diseases with both *in vivo* and *in vitro* study models [52][53]. Numerous studies were done for the purpose of analyzing SFN's means of action. Sulforaphane plays a role in affecting the oxidative stress and antioxidant capacity, neuroinflammation, and several other biological irregularities correlated with autism.

All indication suggests that broccoli, exclusively sulforaphane, and its biogenic foretaste glucoraphanin, is a defensive agent against a diversity of persistent and contagious disorders [54]. Sulforaphane was pronounced in the mid of the past century as an antibiotic and several research have manufactured it since. However, it was firstly isolated from broccoli by the work of Talalay and Zhang, who afterwards indicated its distinctive anti-cancer features [49].

In general, SFN operates by inhibiting phase 1 enzyme, which blocks initiation and changes procarcinogens into ultimate carcinogens. In addition, it stimulates phase 2 enzyme that cleanse carcinogens and accelerates its emission from the body. SFN can subdue cancer through numerous means concerned with the modification of the cell growth's cycle and cell death's pointers.

SFN was firstly recognized in 1992 as a prospective chemo-preventive agent [49], and since then there has been great growth in the number of PubMed citations regarding it.

Many studies shed the light on the stimulation of SFN to Phase-2 enzyme as well as the reticence in the enzymes concerned with the activation of carcinogen. Lately, recent studies have been focusing on the theory that SFN proposes a defense mechanism against the tumor's progress in the "post-initiation" phase and its suppression property.

Sulforaphane has been shown to be an effective antioxidant, antimicrobial, anticancer, anti-inflammatory, anti-aging, neuroprotective, and anti-diabetic agent [6]. Accordingly, sulforaphane appeared to be most effective against colon and prostate cancer but has also been studied for its effects on many other cancers, such as breast (breast cancer stem cells), leukemia, pancreatic and melanoma [7]. Sulforaphane gained special attention due to its ability to simultaneously modulate multiple cellular targets involved in cancer development.

In the year 2013, a quantitative analysis with a group of 69,120 contributors portrayed a primary advance to the connection between nutritional arrays and the progression of cancer. The nutritional arrays reflected distinctive food digestion, and the outcomes of the study proposed a positive shielding effect of natural-like regimen displaying a defensive mechanism against inclusive occurrence of cancer [55].

The current enhancement in receiving phytochemicals, particularly nutritional compounds like sulforaphane, diallyl sulfide, benzyl/phenethyl isothiocyanate, resveratrol, linolenic acid, mahanine, etc., have been studied for their chemo-protective features. Razis et al. explained the function of cruciferous vegetables, especially those composed of a high glucosinolates level such as Brussel sprouts, cabbage, cauliflower, collard greens, kale, kohlrabi, mustard, and rutabaga, in the reduction of various cancer types of cancer: lung, prostate, colorectum and breast [56].

Sulforaphane (SFN) may block carcinogenesis by altering its epigenetic events in the cells; however, it is difficult to deliver it in an enriched and stable form for purposes of direct human consumption [39]. Despite the mentioned benefits of SLF as an antitumor drug in all the literature, its hydrophobicity, sensitivity to oxygen, heat, and alkaline conditions, poor oral bioavailability, and water instability limit its usage in pharmaceutical industries [40].

Based on this fact, researchers sought nanomedicine as a better approach to encapsulate this isothiocyanate compound via nanoparticles and direct its way toward the tumor cells. As it is suggested in the clinical trials database (clinicaltrials.gov), there are currently 61

ongoing studies on sulforaphane, and among them, 22 studies are related to its anticancer therapeutic effect on cancer patients. Examples taken from these trials of different sulforaphane Nano-formulations will be summarized in upcoming sections.

2.5. Biological Effects of Sulforaphane on Cancer Mechanism

Cancer is a complex multi-factorial, multi-step disorder, that manifests itself in uncontrolled cellular reproduction, tissue invasion, and distant metastasis. Carcinogenesis process can be broadly divided into two principal steps, initiation and promotion/progression. The initiation of carcinogenesis involves the induction of an altered but non-neoplastic cell which is capable of participating in the neoplastic process. Promotion/progression includes focal proliferation of the initiated cells and transformation into neoplasia.

Large number of epidemiological studies have reported association between the consumption of SF-rich vegetables and reduction in cancer risk at several sites including the lung (Spitz et al., 2000), bladder (Zhao et al., 2007), breast (Ambrosone et al., 2004), prostate (Joseph et al., 2004), non-Hodgkin's lymphoma (Zhang et al., 2000), and colorectal (Lin et al., 1998; Seow et al., 2002). Such correlation between consumption of SF-rich diet and reduction of cancer risk was found dependent on the number of servings per day/week. For example, consumption of three or more servings of cruciferous vegetables per week was inversely related to risk of prostate cancer, relative to less than one serving per week especially in more advanced case.

Collectively, the reported human epidemiological studies and animal experiments indicate that SF may inhibit the development of different types of cancers during initiation and/or promotion stages (Juge et al., 2007). Several mechanisms have been proposed for the chemo preventive effect of SF. Although early research focused on the "the blocking activity" of SF through induction of phase 2 detoxification proteins, recent studies have recognized several other mechanisms of chemoprevention by SF. These mechanisms include inhibition of phase 1 enzymes involved in activation of procarcinogens, induction of apoptosis, induction of cell cycle arrest, and anti-inflammatory effect (Juge and Traka, 2007). Most likely, these factors interact together to reduce the risk of carcinogenesis.

Sulforaphane is proven to be a highly promising anti-cancer agent due to its impact on the three stages of tumor development (**Figure 2.4**) by the following mechanism of action:

Transformation: DNA fortification by restraining the enzymes that processes the tumor cells and hindering the conflict of mutations [8].

Propagation and Assault: It pauses the growth of these tumor cells (*Phase 2*) and stimulates their programmed demise (*Phase 3*) [8].

Blocking of the advancement of benign lumps to menacing ones (*Phase 2*) and spreading to other parts of the body (*Phase 3*) [9].

Therapeutic property: It is able to preclude, deferral, or antithesis preneoplastic lacerations and undertake on the abnormal cell as a beneficial proxy [10].
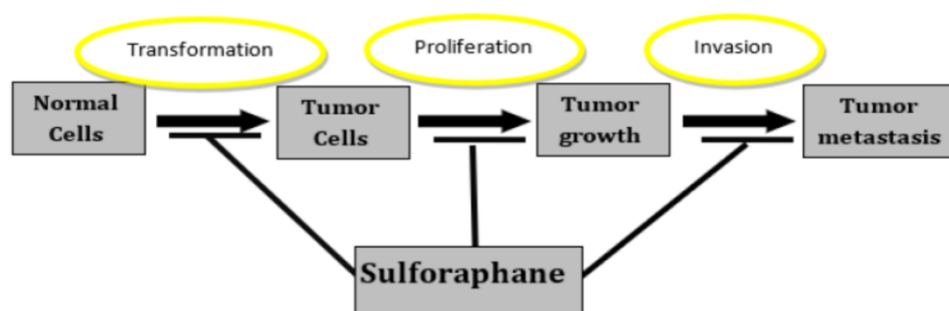
Figure 2.4: Sulforaphane is effective in each: "Transformation, proliferation, and invasion" phases of tumor progression. (As modified from "B.B. Aggarwal et al. in: Turmeric: The Genus Curcuma, 2006, 297-368).

2.5.1. Cell cycle arrest

Hyperproliferation represents one of the cancer's hallmark outstanding for the damage of cell cycle examining occurring. The primary regulators of cell cycle development are cyclin-dependent kinases (CDKs), cyclins, and CDK inhibitors. The phosphorylation state of the compound's various modules and the presence of CDK inhibitors both affect the behavior of CDK complexes. While CDK inhibitors promote cell cycle arrest, cyclin/CDK complexes support cell cycle progression. According to studies, the control of SFN's complex action on various CDKs, cyclins, and CDK inhibitors varies depending on the kind of cell, the amount of therapy, and the exposure time [57].

Cell cycle arrest may play a noteworthy role in SFN's anti-cancer activities, according to in vitro research. Cancer cells from the prostate and colon exhibit G2/M cell cycle halt. The cyclin B/CDK1 complex must be active for this arrest to occur. Wee1 and MYT1 kinases phosphorylate CDK1 in its ATP binding loop, deactivating the complex. Cell Division Cycle 25 (Cdc25), a contrasting phosphatase, removes this restrictive phosphorylation, facilitating the transition into M-phase [57].

The crucial role of cell cycle control in malignant transformation is made clear by an understanding of oncogenesis and apoptosis. Checkpoints are triggered to pause the course of the cell cycle, stimulate DNA repair, or trigger cell death when DNA damage or mutations occur in normal cells. As an illustration, Sundaram et al. demonstrated apoptotic alterations like nuclear condensation, fragmentation, and the development of apoptotic bodies, which were most likely brought on by the activation of NOS2 and NOS3 expression, which led to an increase in nitric oxide. The study also demonstrates that HSP-90AB1, PRKAR1B, ALOX12, PRG3, and NCF2 are upregulated (**Figure 2.5**). On the other side, sulforaphane administration resulted in the downregulation of genes involved in maintaining redox equilibrium, including CCNA1, SOD3, and GPX4 [42].
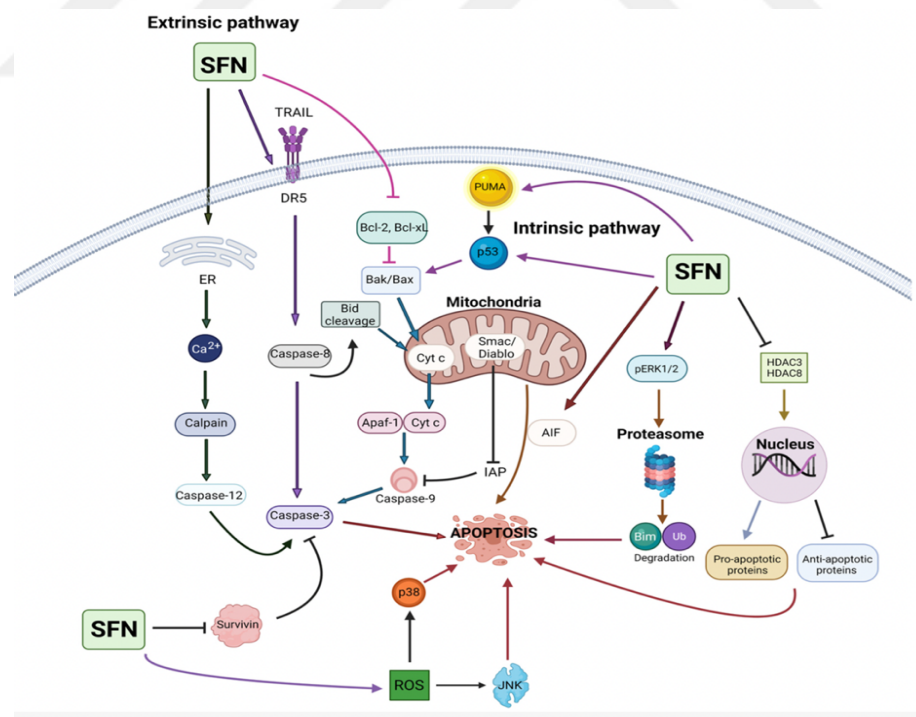
Figure 2.5: Multiple targets in the intrinsic and extrinsic apoptotic pathways are modulated by SFN. Researchers have discovered that SFN can change the expression of or activate/inactivate a number of apoptotic mediators and regulators [53].

However, these regulatory systems are frequently absent in cancer cells, leading to unchecked cell cycle progression and proliferation. Intriguingly, numerous studies have demonstrated that sulforaphane affects the cell cycle in cancer cells. For instance, a cell cycle arrest of at least 18.5% in phase G0/G1 caused by sulforaphane treatment for 24 hours was described by Myzak, Dashwood, and Hao et al. Additionally, the treated group's maximum observed rate was 106% higher than the control groups, and the percentage of cells inducing apoptosis was 43% higher than the control [43] Moreover, sulforaphane mean of action reduced migration and repressed the metastasis development [44].

In a related investigation, 20 m of sulforaphane was used to treat HCT116 cells, which promoted cell cycle arrest and enhanced apoptosis. 24 hours after the treatment, cyclin B1, Cdc25B, and Cdc25C were blocked, which was related to these effects. Sulforaphane does not appear to have any effects on Cdk1, but it was thought that it increased phosphorylation of the Tyr15 deposit on Cdk1, inhibiting the activation of the Cdk1/Cyclin B1 complex. The treatment also triggered the phosphorylation of the Cdc25C's Ser216 residue, and the subsequent binding of 14-3-3 helps Cdc25C move into the cytoplasm [45].

According to other studies, sulforaphane may reduce the expression of cell cycle proteins such Cyclin D1 and Cyclin A in HT-29 cells. On the other hand, it was noted that cell cycle inhibitory proteins including p21 were expressed more. It's interesting to note that sulforaphane therapy was associated with activated JNK, ERK, and p38 as well as the MAPK pathway. Aside from the fact that these pathways and proteins are contradictorily linked to cell growth, the molecular pathway by which they enable the suppression of cell growth is still unexplained [46]. One of the elements that appears to be most associated with sulforaphane therapy is P21 expression. According to transcriptional studies, sulforaphane at a concentration of 50 M facilitates the recovery of p21 levels in Caco-2 cells. In addition, 169 genes showed differential expression in the transcriptomic analysis, with 106 genes showed significant increases in expression and 63 genes displaying a significant drop. This finding suggests that sulforaphane is a promising molecule with a number of different targets that may be researched for a greater understanding of sulforaphane-based cancer treatment [47].

2.5.2. Apoptosis

Apoptosis, also known as the route, where a cell terminates itself when it turns unusual is a foremost mechanism by which cells decease when the DNA is impaired and cannot be restored. Apoptosis is very significant to be able to control the cellular amount and multiply throughout common improvement. When discussing apoptosis, SFN was proved through many studies to be a strong trigger through in vivo studies and in vitro reports [50].

One type of cancer, colon cancer, was the subject of one study that could verify the theory suggested before. SFN decreased the viability of the cells in those HT29, as well as Caco-2 cells. Bcl-2 genes, which are characterized by their ability to revers apoptosis, as well as the gene bcl-x, which carries out the same function, are affected by SFN through negative feedback. Additionally, SFN endorses the caspase-3 and Bax genes, which are known for their pro-apoptotic properties. Additionally, this chemical compound works by starving the polymerase, which is defined as: poly (ADP-ribose) [51]. SFN was applied to UM-UC-3 cells for research, and it worked on them by affecting the mitochondria, changing the place where cytochrome c was housed, and activating the caspases (3 & 9) along the polymer [58].

(SFN) may prevent the development of aberrant cells by interfering with the path they follow within the cells, but it is difficult to provide it in a supplemented and secure formula for commitments to continuous individual intake [59]. Despite the benefits of SLF as an anticancer medicine that have been described throughout the literature, it is ineffective in environments with high O2 levels, even when the temperature rises and the pH of the atmosphere is basic. Additionally, it has a weak ability to spread through blood. Additionally, the practice's water instability limits its use in pharmaceutical processes [60].

2.5.3. Sulforaphane induces apoptosis through mitochondria interreference

The main strategy for treating cancer is to improve the apoptotic responsiveness of cancer cells. A consistent mechanism of action for the effects of sulforaphane administration has been discovered in studies, and it is related to a decreased membrane permeability that induces the death of malignant cells and tumor in vitro models. Studies on cancer suggest that sulforaphane treatment boosts the functioning of the enzyme's caspase-9, poly-ADP ribose polymerase (PARP-1), and cyclooxygenase IV (COX IV), rendering tumor cells

more vulnerable to mitochondria-mediated cell death [55]. It is important to highlight that sulforaphane's apoptotic activity is also controlled through IP3R1, a regulator known to elevate mitochondrial Ca2+ and induce cell death 24 hours following treatment. In addition, after a sustained 7-day treatment period, mice tumors drastically lowered their growth [61].

Surprisingly, colon cancer cells HCT116 and SW480 showed reduced cell success in enhancing the apoptotic effects when Lactobacillus and sulforaphane were combined. The research demonstrates that co-treatment decreased the up-regulation of apoptosis-promoting proteins like TNFR1, cIAP-1, cIAP-2, Bax, and mitochondrial membrane potential, indicating that apoptosis had started [57]. However, Myzak et al. demonstrated that sulforaphane administration for 48 hours caused cell cycle arrest and activated multicaspase activity because of an elevation in HDAC mediated phosphorylation in the regulatory areas of p21 and Bax (**Figure 2.6**) [62].
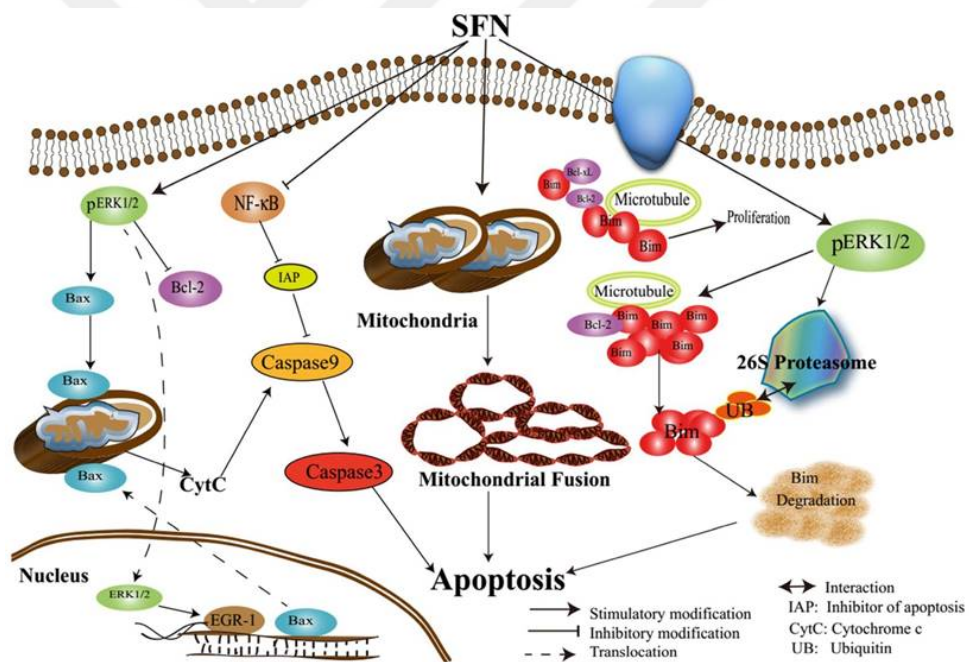
Figure 2.6: Signaling diagram showing how ERK1/2 is used by SFN to cause apoptosis[62].

The activity was also found in H727 and H720 cells that had been treated with sulforaphane, with treated cells displaying cleaved caspase-3 in 70% of cases, caspase-7 in 89% of cases, and cleaved PARP in 113% of cases. After 2 weeks of sulforaphane treatment, 53% of treated cells went through apoptosis [62].

Other experimental studies showed that, at particular in prostate cancer, sulforaphane therapy increases the expression of lysosome-associated cellular membrane 2 (LAMP2), which reduces sulforaphane's ability to cause apoptotic cell death. In PC-3 and 22Rv1 cells treated with 20 mM sulforaphane, LAMP2 knockdown increased apoptosis. The activation of p34cdc2 kinase by dephosphorylation is a last sulforaphane-related mechanism of death; after both 24 and 48 hours, the activation led to apoptosis in 25% and 35% of treated cells, respectively. Additionally, the authors hypothesized that the proteasome must be activated by ubiquitination in order to trigger the process of apoptosis during sulforaphane therapy [63].

2.5.4. Sulforaphane conduct stimulates autophagy

Cells recycle their internal components through the closely regulated catabolic process known as cell autophagy by delivering them to lysosomes. Numerous studies have demonstrated that a wide range of physiological and pathological tasks are carried out by autophagy in cells. When cells can eliminate damaged biological components, ROS and DNA damage are reduced, acting as a tumor suppressor in cancer [63]. A growing number of research show that sulforaphane increases the activation of autophagy in cancer cells. An autophagosome protein called LC3B-II was shown to be elevated in squamous cell carcinoma until it peaked 24 hours after sulforaphane therapy, increasing the formation of autophagosomes and auto-lysosomes. Surprisingly, the reduction in tumor weight observed in in vivo models following treatment could be attributed to the role of autophagy [64].

Similar findings have been reported in regard to the overexpression of LC3-BII in cells treated with 20 M/L sulforaphane. Additionally, after 6 to 9 hours of sulforaphane treatment, microarray analysis revealed 25 genes associated with the autophagy regulation process, with only 13 genes being identified throughout the entire period. These genes include those that are upregulated, such as HSP90AA1, MAP1LC3B, MAP1LC3A, EIF2AK3, HSPA8, and UVRAG, and those that are downregulated, such as ATG4C, FAS, PTEN, ATG10, PRKA [65].

2.5.5. Sulforaphane behavior has undesirable consequences on the PI3K-AKT-mTOR route averting cancer development and proliferation

The PI3K/AKT/mTOR signaling pathway is essential for the initiation and development of cancer and is constitutively active in a number of cancer processes. Second, the PI3K/AKT/mTOR pathway regulates how cancer cells survive, proliferate, migrate, and react to treatment [66]Sulforaphane suppresses the mTOR signaling pathway and promotes autophagy, according to a number of studies [62]. After 24 days of treatment, sulforaphane significantly slowed the growth of tumors in mice by decreasing the phosphorylation of multiple proteins, including AKT, mTOR, ribosomal protein S6 kinase, and eukaryotic translation initiation factor 4E binding protein 1 [67].

Sulforaphane inhibits the PI3K/AKT/mTOR signaling pathway, which when combined with chemotherapy drugs like acetazolamide causes apoptosis in a synergistic manner. In comparison to either in vitro or in vivo xenograft tissues, this combination decreased cancer cell survival. Since both H727 and H720 cell treatments were associated with the activation of apoptosis, an increase in the p21 cell cycle inhibitor, and downregulation of the pathway, the PI3K/Akt/mTOR pathway (**Figure 2.7**) is a primary target of the acetazolamide + sulforaphane combination treatment [62].
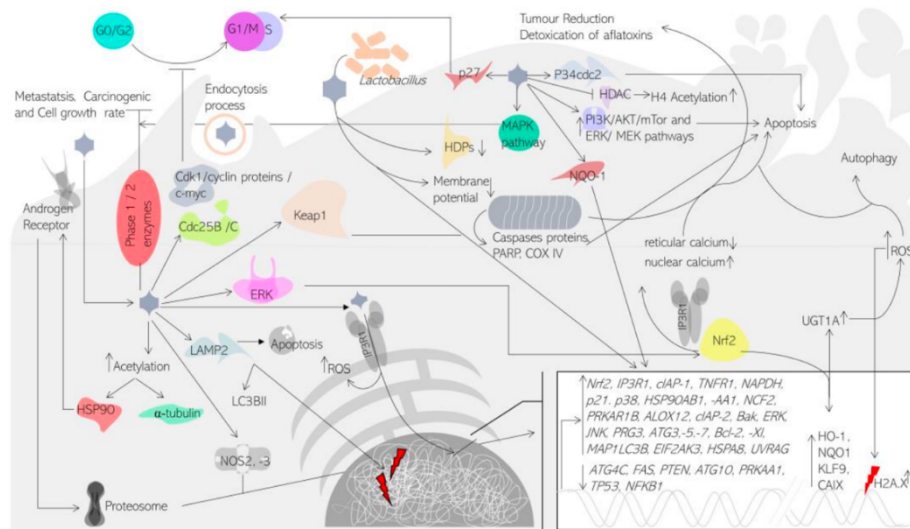
Figure 2.7: Molecular mechanisms involved in the treatment of sulforaphane. Sulforaphane's molecular targets have the potential to enhance the effectiveness of cancer treatments by inducing cell cycle arrest, DNA damage, autophagy, and/or apoptosis.

Angiogenesis, which produces solid tumors that are highly vascularized and have a high density of micro vessels, is regarded to be essential to the development and spread of

cancer. Recent research suggests that signaling cascades between tumor cells and the stromal environment are involved in tumor angiogenesis. As a result, abnormal vasculature develops, which promotes the tumor's development and spread.

Davis et al. suggest a different mechanism for the sulforaphane's anti-angiogenic effect. Their findings indicate that FOXO activation causes cell migration and inhibits capillary tube development [68]. Additionally, angiogenesis contributes to the worsening of hypoxia by promoting the expression of hypoxia-inducible factor 1 (HIF-1), a protein linked to both angiogenesis and cancer. It controls a large number of genes, among them vascular endothelial growth factor (VEGF), inducible nitric oxide synthase, and lactate dehydrogenase A. Surprisingly, sulforaphane has been shown to increase JNK and ERK signaling, which reduces hypoxia-induced HIF-1 expression.

2.6. Cancer-Subduing Outcomes of Sulforaphane in Breast Cancer

As shown in (**Table 2.3**), as seen in other cancer types, sulforaphane's effect mechanism in the breast carcinoma case is multi-targeted and varies from apoptosis to autophagy.

Table 2.3: Sulforaphane induced effect in several breast cancer cell lines [56].

Subjects	SFN Dosage	Anticancer Effect	Genes Targets	Ref.
MDA-MB 231.	40 μm	Induction of Apoptosis	ZEB1, Claudin, and Fibronectin	[62]
MC7-cells and MDA-MB-231	5.0 μM	Cell-cycle blockage	CCND1 and CDK4	[69]
MCF-7 and MDA-MB-231	16.64 M	Cell-cycle blockage	No data available	[70]
MDA-MB-231, SK-BR-3, and MCF-7	20 ng/mL	Cell cycle, overpowering of osteolytic bone reabsorption	RUNX2 and NF-κB1	[71]

In breast cancer, sulforaphane's anticancer activity appears to be dependent on the concentration administrated. For illustration, 40 M sulforaphane promotes the induction of early/late apoptotic and necrotic cells in MDA-MB 231 cells. Stream of genes involved in the endothelial transformation, including such ZEB1, claudin, and fibronectin, was seen at dosages of 20, 30, and 40 M at 72 hours after sulforaphane therapy [62].

Table 2.4: Sulforaphane nano-formulation induced effect in several breast cancer cell

Subjects	Sulforaphane dosage	Anticancer Effect	NPs	Ref.
MDA-MB-231 and MCF7	487.5 mg/g + 9.375 mg/g of DOX	Hinder tumor cell development, ROS production, mitochondrial destruction	Liposome NPs	[72]
MDA-MB-231 MCF-7	8.7 uM and 500 nM of (PTX)	Withholding of the proliferation of the cell	Emulsion NPs	[73]
MCF-7	48.97 uM and cisplatin at 72.59 uM	Initiation of apoptosis over DNA impairment.	Metaxy poly(ethylene glycol)-poly NPs	[74]
MCF-7 and BT-474	48 nM and metformin 42 nm.	Cell development blockage	Carbon NPs	[75]

Castro et al. observed a comparable effect on MDA-MB-231 at 15 M of sulforaphane with a 45% reduction in cell proliferation (**Table 2.4**

Table 2.4). Additionally, five weeks of therapy with 50 mg/kg. The study also found that stem cell-related genes like aldehyde dehydrogenase 1A1, sulforaphane resulted in a 29% decrease in tumor volume in BalbC/nude mice lines [76].

NANOG, GDF3, and After 36 days of therapy, the tumor transcriptomes showed growth inhibition of the gene forkhead box D3, which maintains embryonic pluripotency [77].

Subjects	Sulforaphane dosage	Anticancer Effect	NPs	Ref.
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MCF-7 and BT-474	48 nM and metformin 42 nm.	Cell development blockage	Carbon NPs	[75]

In line with earlier research on the impact of sulforaphane on the cell cycle, in two different breast cancer cell lines, MC7-cells and MDA-MB-231 cells, Royston et al. saw the same outcome. The study found that Cyclin1 and CDK4 expression were downregulated, which resulted in a stoppage of cell cycle progression in the G1/S phase [57].

Sulforaphane's effects vary liable on the cell line reviewed, with the IC50 for three different lines being 4.05, 19.35, and 16.64 for MCF-7, MDA-MB-231, and SK-BR-3 cells, correspondingly. There have been statements of similar results, such as the halting of cell cycle and the assembly of reactive oxygen and nitrogen classes. Furthermore, MDA-MB-231 cells was disturbed by sulforaphane treatment, it caused DNA breakage on both the double-strand and single-strand sides (**Figure 2.8**) [70].
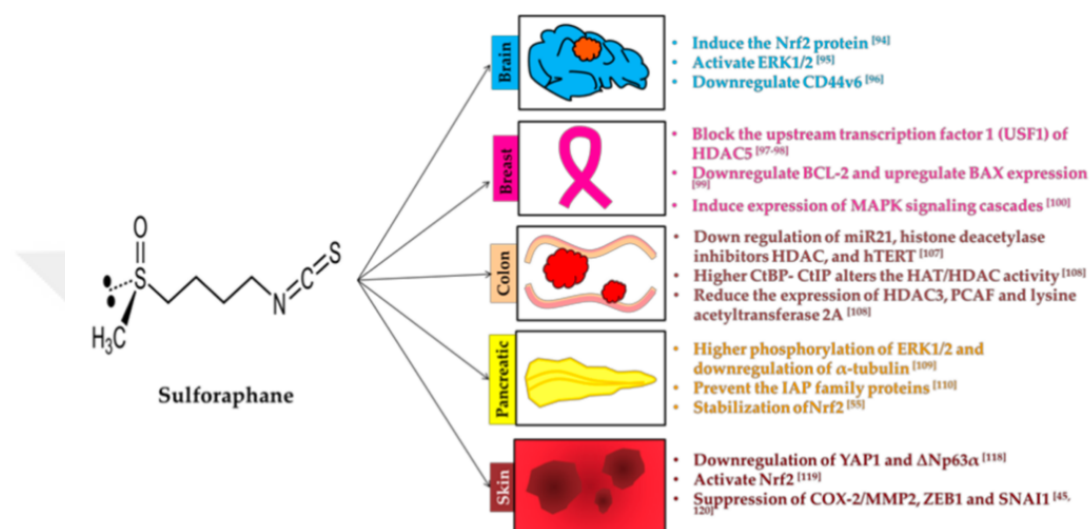
Figure 2.8: Biological effects of SFN on cancer mechanism [78].

Last but not least, treatment with sulforaphane has the ability to stop the "vicious spiral" of osteoclast development that frequently occurs in breast cancer. The NF-B 1 gene is increased as a result of sulforaphane's ability to block the transcription factor RUNX2, according to the results. This shows that sulforaphane influences the NF-B pathway's regulation indirectly. The same research team used in vivo models to confirm these findings and discovered that the plasma levels of certain proteins such CTSK, RANKL, and IL8 decreased by 30 to 52 percent [79].

2.7. Nanomedicine Applications of Sulforaphane

2.7.1. Cancer nanotechnology: novel era of successful targeted treatment

Nanotechnology has updated cancer treatment and diagnosis methods over the past few decades. A remarkable advancement in the fight against cancer Pharmacokinetics are improved by nanomedicine (reliability, aqueous-solubility, and bio-availability). For the

treatment of cancer, the FDA has granted approval the use of multiple nanoparticles, comprising polymeric micelles, liposomes, albumin, and numerous others [80].

High drug loading capacity, a defined targeting of tumor positions by avoiding non-specific cellular comprehension, advanced drug bioavailability, and amplified imaging sensitivity are many of the few features of operative successful nanomedicine. Furthermore, nanomedicine has the capacity to deliver biomolecules, such as peptides, nucleic, and therapeutics, to the intended site accurately, hence enhancing their usefulness. A "smart drug" significance relies greatly on site-specific accumulation and prodded drug release. There are number of distinctive kinds of nanomedicines that are receptive to a trigger parameter, including reaction to pH, its oxidative state, and reaction to various enzymes. The tumor microenvironment is thought to be the foremost key to take into consideration when drug synthesizing is in process. The acidic microenvironment for example can be targeted through altering the pH parameter of the smart drug which could lead to a burst behavior when it reached the specific site [69].

For the treatment of cancer, the majority of therapeutic nanoparticles are given systemically. However, a number of biological events, including opsonization, extravasation, and interaction with the perivascular tumor microenvironment, tumor tissue penetration, and tumor cell internalization, can affect the systemic administration of nanoparticles. Another element that may disturb biological processes is the features encompassing the nano-formulation (size, charge, porosity, flexibility, mechanical and electrical properties, etc.). Nowadays, nanomedicine boosts custom methods of delivery to specific cells through the means of release of the drug into the targeted active site. Antibodies, their fragments, and many growth factors can be modeled and decoded on the outer shell of the nano-formula encapsulating the anti-tumor compound in order to achieve an enhanced targeting profile [72].

Additionally, targeting molecules can be exerted into the nanoparticle outer-surfaces to purposely categorize evident receptors or antigens that are widely found in a cancerous cell. There are many active targeting nano drug delivery systems are still pending for their approval through the FDA to be used clinically, although considerable number of formulas are still in the clinical trials stage [42].

Since nanoparticles participate with a crucial role in drug delivery, nanotechnology has been broadly investigated and expended in the management of cancer. An increase in the

stability of drug, its compatibility in biological environment, its upgraded absorbency and retaining features, and a detailed mean of targeting are all related to the advantages offered through nanomedicine when compared to chemotherapy and conventional medications used over the past decades for the cancer management and treatment. It is estimated that the application of nanoparticles will bypass the main downsides of sulforaphane, comprising its insolvability in water and bioavailability.

New analyses of sulforaphane's encapsulation inside a nano-complex vehicle have produced favorable results based on that theory. For instance, a number of nanoparticle applications have recently been newly documented for their promising Sulforaphane delivery to breast cancer cells. These nanoparticles include: Fe_3O_4 coated with gold [81], selenium [82], tellurium [83], PEGylated Fe_3O_4 [84], monomethoxypol, and Fe^{2+} and Fe^{3+} based nanoparticles [85].

The results of these research suggested that employing nanoparticles can reduce the viability of tumor cells including MDA-MB-231, MCF-7, and SKBR-3. These investigations embolden tumor cells to trigger BAX and Bak (pro-apoptotic) and to reduce the expression of bcl-2 and bcl-xL; thereby, it fastens the initiation of apoptosis. Additionally, those studies' MTT analysis exhibited that selenium and tellurium nanoparticles are directed toward the abnormal cell with a specifc manner and pathway that favorably has no impact on healthy cell lines like MCF10-A [72], [82], [83].

The research thus demonstrates that sulforaphane-coupled nanoparticle therapy has a trustworthy favorable anti-cancer impact; therefore, its administration in sequencing with additional chemotherapeutic drugs through nanoparticles provides a significant potential for future studies.

2.7.2. Possible outcomes of sulforaphane-nanoparticles in literature:

According to research done on breast cancer cell lines as MCF7, a concentration of 487.5 mg/g of sulforaphane and 9.375 mg/g of DOX were entrapped in liposome nanoparticles. Cell viability was statistically diminished subsequent to the incorporation of the of DOX/sulforaphane nanoparticles. When joined, sulforaphane and DOX also result in a total of fifty percent drop in cell viability, and it is demonstrated to be beneficial at hindering the development of tumor cells (**Figure 2.9Error! Reference source not found.**). Additionally, sulforaphane and DOX incorporation into nanoparticles induce

several primary mechanisms that were previously explained in Chapter 1 including: ROS production, mitochondrial destruction, and autophagy [72].
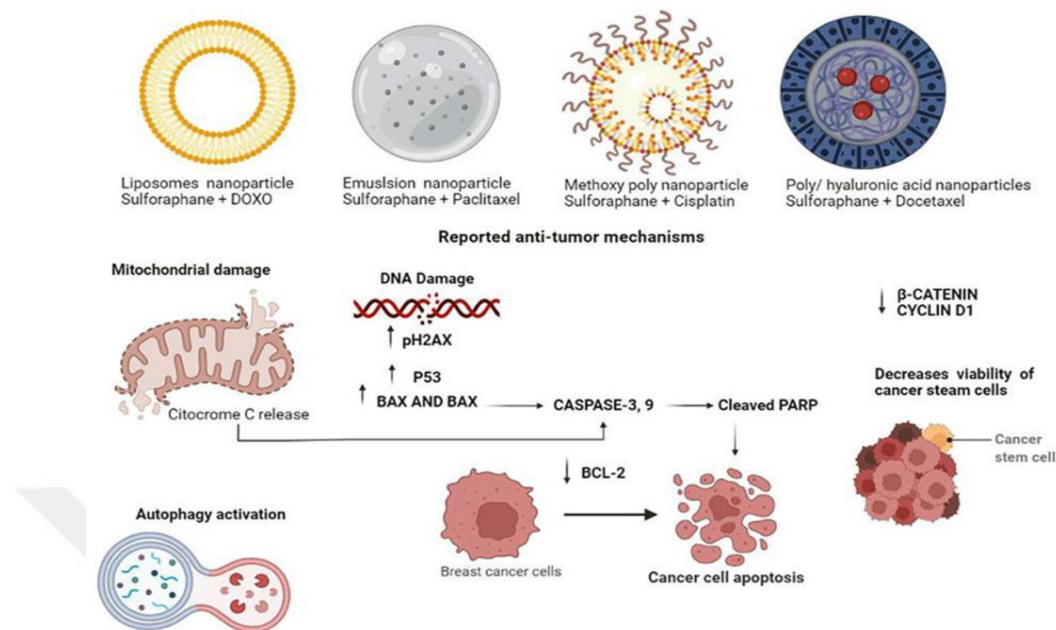
Figure 2.9: Sulforaphane's anti-tumor actions. Mechanisms for the loading of poly (D, L-lactideco-glycolide)/hyaluronic acid nanoparticles with liposome nanoparticles, methoxy poly (ethylene glycol)-poly (lactide-co-glycolide), and poly (ethylene glycol) nanoparticles[80].

The excipients in PTX, a highly successful chemotherapeutic treatment for breast cancer cells, are responsible for a multitude of adverse effects. Sulforaphane, on the other hand, lowers the quantity of excipients utilized in commercial PTX preparations [86].

However, it has been found that via enhancing the binding to DNA, the methoxy poly (ethylene glycol)-poly (lactide-co-glycolide) nanoparticle loaded with sulforaphane at an approximate of 50 M raises the sensitivity of cisplatin at a concentration of 70 M. Cisplatin discreetly induced apoptosis in MCF-7 cells with an apoptotic rate reaching 20%. In contrast, when sulforaphane and cisplatin nanoparticles were exploited side by side, the apoptotic frequency amplified to a value of 40%.

The principal mechanism of action is apoptotic induction through the elevated illustration of specific gene p-H2AX, p53, cleaved PARP, and Bcl-2 that was induced by the sulforaphane + cisplatin nanoparticle. The development of tumors in *in-vivo* prototypes followed by administration of sulforaphane & cisplatin nanoparticles was reduced by 74.1% [87].

Previous research has shown that poly (D, L-lactideco-glycolde)/hyaluronic acid nanoparticles encapsulating DTX and sulforaphane have a unique effect over breast cancer stem cells. Following 72 hours of treatment, sulforaphane & DTX nanoparticle diminished the viability of MCF-7 cancerous cells through the targeting the CD44 and CD24-epithelial-precise antigen. Mutually b-catenin and cyclin 1 levels were reduced, causing an inhibition of cell proliferative mechanics.

In modern times, NPs have released positive prospects to improve original drug delivery methods suitable to their prospective biomedical solicitations. Previous trials demonstrated different types of nanoparticles that were used to aid Sulforaphane's delivery [59].

Previous trials demonstrated different types of nanoparticles that were used to aid Sulforaphane delivery. For instance, magnetic nanoparticles enhance the enactment of cancer management because of their numerous advantages containing the tremendous paramagnetic character, limpness, affluence of discovery within the individual's form, and elevated biological fit inside the human body, in which it doesn't react negatively with the rest of the chemical feedbacks going on [60].

Nevertheless, the iron oxide NPs can be effortlessly dissolved in the open, so a grafting policy should be conveyed out. In one of the trials, Sulforaphane was loaded in Fe3O4 with an inner shell of gold nanoparticles. The encapsulated SFN caused a noticeable shrinkage in cell feasibility and provoked cell automatic death. The acquired outcomes of the MTT analysis of the MCF-7 cells revealed that SFN encapsulated within the mentioned formula exposed a severe influence on the tumor cells when we compare it to the execution of free SFN [43].

In one of the trials to improve SFN's efficacy, a consistent micellar carriage system expending (mPEG-PCL) was reliable to be investigated. The results advocated that SFN/mPEG-PCL micelles can be an applicable breast cancer treatment approach in

prospect because the study attested that the SFN-loaded mPEG-PCL micelles prompt at all concentrations were considerably cytotoxic in the case of MCF-7 cell line [88].

In another study, Sulforaphane's anti-tumor effect was tested by binding it to gold nanoparticles, the results obtained proved the gold nanoparticles added the advantage of better stability, higher cytotoxicity against cancer cells, and enhanced permeation through

GIT [89]. Below, a table is given to summarize several articles that were concerned with SFN drug delivery systems (**Table 2.5**).

Table 2.5: Sulforaphane based nanomedicine for cancer found in the literature.

Drug Delivery System	Achievements	Limitations
Polymer NPs: Micelles	- Shape and size: unvarying and round sphere SFN-loaded in micelle. Size: 107 nm. - EE of SFN: 86 ± 1.58% - In-vitro release of SF: Outstandingly persistent - Cytotoxicity: Expressed high toxic levels in MCF-7 cells - Apoptosis & Cell cycle arrest: Real-time PCR and flow cytometry were utilized to validate that the SF-loaded micelle could be competently prompting apoptosis in MCF-7 cell line.	- The micelle expressed low cytotoxicity in the case of MCF-7 cell line, which could demonstrate that the following nano-carrier could also be cytotoxic against the healthy human cell lines but there was no experimental data to support the following theory, which in its turn leaves it vague. - The data provided by the article was limited. It wasn't a detailed study in aspect of the effect of the following drug delivery system over the healthy cell-lines.
	- Mw of the copolymer: 20.4 KDa. - Shape and size: A homogeneous spherical shape. - EE of SFN-NP: 87.1 ± 1.58%. - In-vitro release of SFN: No early eruption of SFN was perceived - Apoptosis: SFN-micelles amplified the apoptosis induction and was further justified by the triggering of caspases-9 and inhibiting of bcl-2.	- A significant alteration in the zeta potential from -2.01 mV (micelles) to -7.57 mV (SFN-loaded micelles). - The micelle size investigated by AFM was 107 nm, a bit lower than that demonstrated by DLS, because of the distorted micelles following the evaporation of water. - The obtained SFN-micelles were unstable in water, they aggregated. - The size of all micelles amplified throughout after the passage of 30 days. The copolymer was observed to swell.
Magnetic and gold NPs	- Size of SFN-NPs: 40 nm. - Zeta Potential is: 9.47±4.6 mv, which is applicable for cell membrane penetration. - In vitro release: The release of SFN from the NP at low pH level is higher than the eruption of SFN form the NP at the higher pH values. - apoptosis induction and was further justified by the inibition of bcl-2 and bcl-xL genes.	- The free magnetic NPs can be quickly oxidized when exposed to air, the gold grafting procedcure is a necessary. - The NPs exposed heterogeneity which could influence the NPs bio-scattering and directing. - Cytotoxicity: Cell viability reduction didnt exceed 50% for the SFN-loaded NP.
	- Size of SFN-NPs: 30 nm. - Loading capacity: ~72% of SFN was loaded. - In vitro release: stable controlled retention ~72% release of SFN over 120h.	- The free magnetic NPs can be quickly oxidized when exposed to air, the gold grafting procedcure is a necessary. - The NPs exposed heterogeneity which could influence the NPs bio-scattering and directing.

A small number of nano formulations have been created to address the aforementioned problems and improve their anti-cancer properties. In this regard, Mangla et al. created nanostructured lipid carriers with tamoxifen and sulforaphane loaded on them that increased intestinal permeability and the oral bioavailability of TAM and SFN by 5.2-fold and 4.8-fold, respectively. Sulforaphane was found to lessen the harmfulness connected to TAM, according to in-vivo studies [90].

Huang et al. demonstrated in a different study that Docetaxel and Sulforaphane loaded into PLGA/hyaluronic acid nanoparticles effectively prompted cytotoxicity and diminished breast stem cells percentage by downregulating catenin expression in vitro. Additionally, they discovered that these nanoparticles were more effective than free drugs at inhibiting breast stem cells growth and self-replenishment in vivo models [91].

Based on all the information presented by this literature and the results obtained from several recent clinical trials, it is deduced that vesicular drug delivery systems such as liposomes, emulsions, niosomes, proniosomes, solid lipid-Nano particles, ethosomes, nanoparticles, etc. have gained more attention as they enhance the drug's anti-tumor effect. But emulsomes, in specific have rose as a system, which bypasses many disadvantages associated with other systems, developed as novel lipoidal vesicular system with internal solid fat core surrounded by phospholipid bilayer. This technology is designed to act as vehicle for poorly soluble drugs or compound such as SFN. The compound is enclosed in the emulsomes and provide prolong existence of drug in systemic circulation [92].

Table 2.6: Sulforaphane based nanomedicine for cancer treatments [83].

S.no	Nanotechnology tool	Size	Route of administration	Clinical /In-vivo results
1.	SFN encapsulated within a nano lipid carrier [93]	145.38 ± 4.46 nm	Oral	The analysis demonstrated that the relative bioavailability of the nano formulation was relatively higher than that of the free SFN suspension.
		121.9 ± 6.42 nm	Oral	NLCs improved the drug's permeability while also boosting SFN's oral bioavailability.
2.	SFN – loaded into a Copolymer-Based Nanoparticles	1793±28 nm	Intravenous	It expressed an anti-cancer activity by inhibiting tumor growth and self-renewal in MCF-7 when compared to conventional methods [91].

2.8. Emulsomes

Emulsomes represent lipid-based drug delivery systems with broad variety of therapeutic applications principally for drugs that are poor water soluble. Emulsomes are tiny lipid assemblies with cores that are able to carry the pharmaceuticals who are insoluble in water without the need to any additional solvents or moieties. Emulsomes are considerably more stable, ranging within the nano-size, and might be used for the intravenous route when compared to already established vesicular formulations. In addition, emulsomes might offer a more affordable alternative to the lipid formulations currently used in the market [94].

Emulsomes are a novel and new lipoidal vessels that consists of a phospholipid bilayer incorporating a solid fat core inside as presented and illustrated in **Figure 2.10**. Emulsome is composed of a soya lecithin and solid lipid core element that is steadied through the addition of cholesterol in the synthesizing procedure. To create emulsomes within the acceptable range, the formula is loaded and then sonicated [92] as applied and explained in **CHAPTER 3.** The polymer that is retained as the center should be solid at 25°C. The emulsomes were stabilized in the shape of an Oil/Water emulsome due to the elevated soy-lecithin concentration. These lipid particles with fat cores gets scattered in an aqueous

phase which certify its usage in cancer treatment taking into consideration the cancer microenvironment [94].
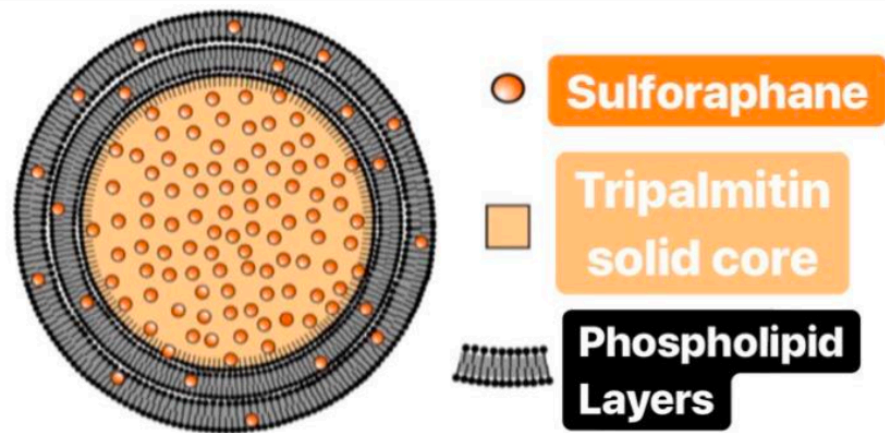
Figure 2.10: The scheme of an Emulsome formulation (As modified from Mehmet H Ucisik1, Seta Küpcü1, Bernhard Schuster1 and Uwe B Sleytr, et al/2013).

Emulsome is a lipid-based drug delivery method with an extensive range of beneficial practices, especially for water-insoluble compounds[26]. Emulsomes are lipid assemblies with cores that transport water-insoluble drugs without the requirement for an additional surface attachment or another solvent. In comparison to other formulations that are also physically shaped as vessels, emulsomes shine in that area in which they are much more stable, and their nano-size is very crucial (**Figure 2.11**). Thereby, it is a novel, emerging delivery system, that could demonstrate a fundamental role in the resourceful treatment of transmissible diseases such as: hepatitis, HIV, Epstein-Barr virus, hepato-splenic moniliasis and primeval leishmaniasis in precise [14], [19], [39], [41]
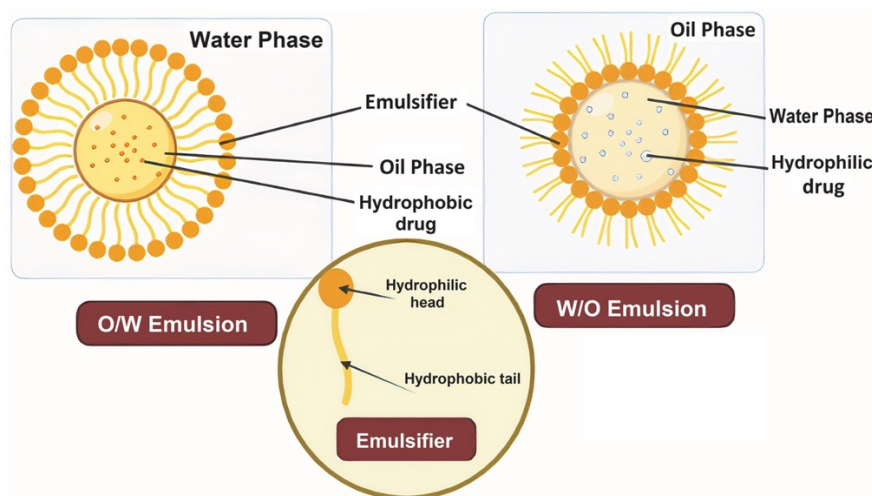
Figure 2.11: Structure of an o/w and w/o nano emulsions [94].

2.8.1. Emulsomes as a drug delivery system

Additionally, the systems can be used in systemic fungal infections to quickly load reticuloendothelial system organs with the integrated medicine for the total eradication of the intracellular pathogens. Emulsomes may improve the controlled oral administration of drugs and biomolecules. It's because they come in sizes ranging from micro to nano and can be administered through the intravenous route. A more reasonable alternative to the marketable lipid formulations now used to treat viral and fungal infections may be emulsomes. Emulsomes stipulate a controlled and prolonged release of medicine. When associating them to liposomes, which have a release that is only prolonged by six hours, emulsomes give a better drug release profile that is prolonged by up to 24 hours [40].

Emulsomes are nanoscale in size when compared to other vesicular delivery systems like niosomes, pharmacosomes, and ethosomes. Because of their smaller size, they are the best carriers for both oral and intravenous drug delivery and can increase a drug's bioavailability. Emulsomes are estimated to behave like a type of natural lipoprotein located in the body, due to their structural similarities which favors its usage in medicine. Through the enterocytes of the GIT tract, these lipid-structured particles are consistently absorbed through an endogenous lipid absorption progression [95]. The coordination of apolipoprotein and lipid synthesis, as well as their intracellular assembly into mature lipid-containing particles, are all crucial components of the intricate process of long-chain triglyceride absorption from enterocytes [92].

Monoglycerides and free fatty acids are the primary triglyceride breakdown products. Passive diffusion is expended to absorb these into the enterocytes, where they are then transferred to the endoplasmic reticulum (ER), where complex lipids are biosynthesized to form triglycerides.

The ER and Golgi apparatuses are responsible for the synthesis of prechylomicrons, the precursors of chylomicrons. In the ER, Lipoproteins are created and then delivered to the Golgi. The chylomicrons are exocytosis, releasing the triglyceride-rich lipoproteins into the intercellular space, once they have reached the lateral membrane of the enterocytes [96]. Through a number of physiological developments, such as hindered gastric discharging, lipid excipients can disturb oral intake. Through increasing the fluidity of the membrane lipids by promoting bile stream and pancreatic juice excretion, or by

directly influencing enterocytes, which are in charge of drug passage and clearance [29], [40], [90].

2.8.2. Advantages of emulsomes

Emulsomes shield the medication from the unpleasant gastrointestinal background of the stomach since it is trapped in the triglyceride lipid core prior to oral delivery. This theory might be supported by the fact that stomach enzymes and pH cannot dissect triglycerides. Emulsomes enhance the bioavailability and dissolution of drugs that are not well soluble in water. Triglycerides make up these micelles, which are arranged as lipid bilayers with the hydrophilic head cluster opposite to the water on each side and the hydrophobic ends lined up next to one another. Phospholipids are employed most effectively as excipients for drugs that aren't highly water soluble because of their special characteristics. Lipid is the primary component of emulsomes. Oral controlled medication delivery systems are made with lipids.

It is uncommon to employ lipid-based methods that increase drug exposure. Emulsomes make the already used commercialized lipid preparations more inexpensive by slowing down the rate of medication use. Emulsome-based technology displayed remarkable targeting potential.

The formulations have the potential to greatly lessen toxicity problems brought on by the medication's complimentary confinement in cell membranes by extending the time the drug is in effect at relatively low concentrations. They significantly change how drugs are metabolized. They also prevent the emergence of multi-drug resistance, which is typically associated with the overexpression of a cell membrane glycoprotein, which causes the medication to be effluxed from the cytoplasm and concentrated ineffectively inside the cellular compartment.

2.8.3. Applications of emulsomes

2.8.3.1. Drug targeting

Emulsomes' capacity to target medications is one of its most advantageous features. Drugs can be directed to the reticulo-endothelial system via emulsomes. Emulsomes are taken up preferentially by the reticulo-endothelial system. Opsonins, circulating blood components, regulate the intake of Emulsomal vesicles. These opsonins identify the vesicles and signal their clearance. Such medication localisation is used to treat animal

malignancies that are known to spread to the liver and spleen. This medication localisation can also be utilized to treat liver parasite infections [74]. Drugs can be directed at organs other than the reticulo-endothelial system using emulsomes. Vesicles can be directed to particular organs by attaching a carrier system (like antibodies) to them (**Figure 2.12**) [82].
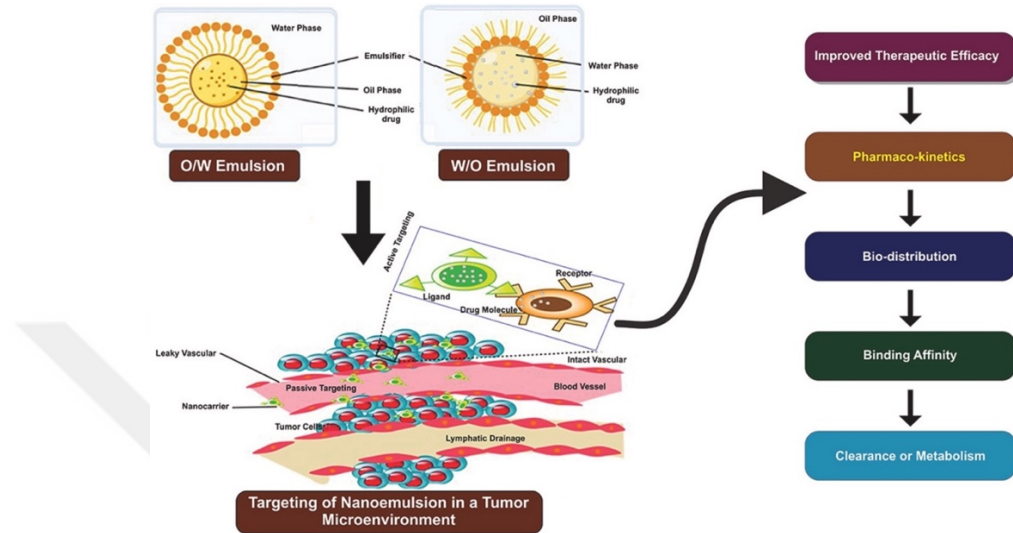
Figure 2.12: A review of the uses of chemotherapeutic-loaded nano emulsions in the treatment of cancer [92].

2.8.3.2. Anti-neoplastic treatment

Serious side effects are a common occurrence with antineoplastic medications. Emulsomes can change a drug's metabolism, increase its circulation, and extend its half-life, all of which reduce its negative effects. Emulsome entrapment of methotrexate shown advantages over the unentrapped medicines, including a slowed rate of tumor development and higher plasma levels with slower clearance (**Figure 2.13**).
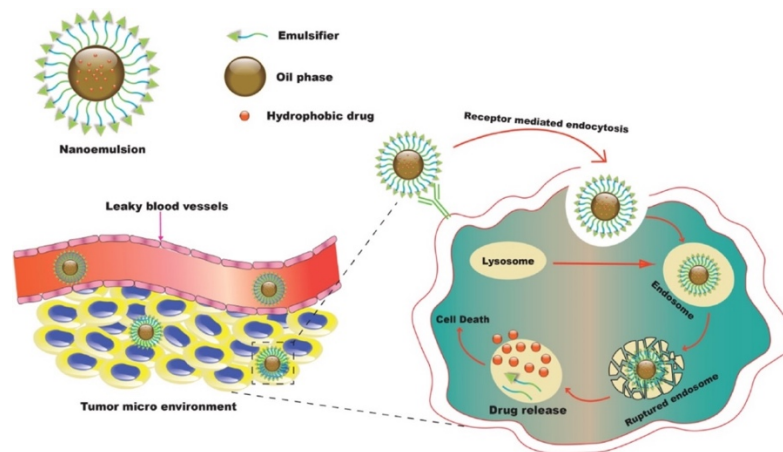
Figure 2.13: Uptake of targeted nano emulsions by the cancer cell [66].

2.8.3.3. Leishmaniasis

A parasite from the genus Leishmania infects the liver and spleen cells to cause leishmaniasis, a disease. Antimony derivatives (antimonials), which can harm the heart, liver, and kidneys in higher amounts, are frequently prescribed medications for the condition. The use of emulsome in experiments revealed that it was able to provide bigger doses of the medication without inducing side effects, allowing for increased treatment efficacy.

2.8.3.4. Used in biotechnology

Due to their immunological selectivity, low toxicity, and higher stability, emulsomes are used in the study of immune response. They are being used to research the type of immune reaction triggered by antigens. Estradiol from vesicular formulations permeating through human cells in vitro.

CHAPTER 3

3. EXPERIMENTAL PART

3.1. Materials

R-Sulforaphane (High purity, 50mg), Glyceryl tripalmitate (tripalmitin, purity ≥99%), 1,2-dipalmitoyl rac- glycero-3-phosphatidylcholine (DPPC,99%), and Cholesterol (95%, stabilized 25G), Ethanol (%), Chloroform (≥99.8%), were purchased from Sigma-Aldrich GmbH, Germany. FITC Annexin V/Dead Cell Apoptosis Kit with FITC annexin V and PI, was purchased from ThermoFisher Scientific.

Materials used for cell culture media: PBS, EMEM, FBS/FCS, and Antibiotics.

3.2. Methods

3.2.1. Synthesis of sulforaphane-emulsomes

Tripalmatin, DPPC, and Cholesterol with a weight ratio 41:2:4 were dissolved in 1.5 ml chloroform. Sulforaphane dissolved in 50 μl ethanol was added separately to the obtained mixture. Both solutions were mixed and the organic solvent was completely removed using a rotary evaporator (BUCHI Labortechnik AG, Büchi, Switzerland) under reduced pressure at 474 mbar and 54°C. The formed dry film was hydrated with double distilled water, the temperature was set to 80°C and the solution was rotated until the lipid film was resuspended.

After the passage of total four hours, the flask containing the mixture was transferred into the Sonicator (Bandelin electronic, Berlin, Germany) for one hour. The obtained emulsome suspension was placed in ice for 10 min. SLF-Emulsome preparations were centrifuged at 11,000 rpm for 5 minutes to spin down unincorporated sulforaphane. The SLF-Emulsome suspension, i.e. the supernatant, was stored at 4°C until further

characterization and cell culture studies. Empty emulsomes were prepared as described above but without sulforaphane.

3.2.2. Characterization techniques

3.2.2.1. Zetasizer analysis

By using the Zetasizer Nano ZS (Malvern Instruments Ltd., UK), SLF-emulsomes that had been diluted in 1 mM KCl solution (pH 6.3) and had a final DPPC concentration of 4 g/ml were examined for their particle size distribution (Dynamic Light Scattering; DLS) and zeta potential characteristics [97].

3.2.2.2. Scanning elector microscopy analysis

The size and shape of emulsome formulations were analyzed using a scanning electron microscope (Zeiss EVO-HD-15). A short-term fixation pre-treatment method was used before the imaging process. In a nutshell, emulsome samples were put onto an aluminum holder and allowed to dry at 4°C. The dried samples were fixated for 15 minutes using PBS containing 2.5% glutaraldehyde. Following the removal of PBS containing 2.5% glutaraldehyde, samples were washed three times in total over the course of 10 minutes with distilled water. The samples were subsequently subjected to gold-sputtering (EM ACE200, Leica), followed by SEM analysis.

3.2.2.3. Confocal laser scanning microscopy

To further examine how emulsomes behave as they disperse in an aqueous environment, confocal laser scanning microscopy (CLSM) study was carried out in addition to SEM analysis. A sample of 5 l of emulsomes was placed on a glass for CLSM analysis (LSM780, Zeiss, Turkey).

3.2.2.4. Quantification of sulforaphane-emulsomes

HPLC was used to analyze the SFN-Emulsomes' chemical makeup. To eliminate water from the system, emulsomes were dried in a vacuum evaporator for two hours. The concentrated emulsion was next mixed in ethanol at a ratio of 1:10 to dilute it. Centrifugation at 18800 g for 40 minutes precipitated the matching diluted emulsions. The supernatant was centrifuged, vortexed, and then put right into the HPLC apparatus. Briefly stated, 10 l of the sample was automatically injected into the injection port and analyzed on a C18 column at 33°C using a 40:60 (v/v) ratio using a Nucleosil 120-3C18,

1504 mm, Macherey-Nagel, Germany [51]. Ultraviolet detection at 420 nm using UV/VIS-Detector UVD 170U/340U was used to measure the quantity of SFN (Dionex, Germany). The peak area that was correlated with the standard curve was used to establish the compositional distribution of sulforaphane in the sample. Sulforaphane, DMC, and BDMC all eluted at retention times of 17.3, 15.4, and 13.7 min, respectively, during the 20-minute total HPLC analysis time per sample [97].

3.2.3. Cell culture studies

3.2.3.1. Cell lines

MCF7 (Human Caucasian breast cancer cell line) was purchased from the Department of Genetic Engineering at Yeditepe University (Istanbul, Turkey). At room temperature, MCF7 cells were grown in a medium made up of EMEM with 2mM Glutamine, 1% Non-Essential Amino Acids, 10% Foetal Bovine Serum FBS/FCS, and 1% antibiotic/antimycotic (both PAA).

Cells for MCF10A (human breast epithelial cell line) were received from the Department of Genetic Engineering at Yeditepe University (Istanbul, Turkey). For SILAC, they were grown in (DMEM)/F12 without L-lysine and L-arginine. At 37 degrees Celsius in a humid environment with 5% CO_2, the medium was supplemented with 5% horse serum, 20 ng/ml epidermal growth factor, 0.5 g/ml hydrocortisone, 100 ng/ml cholera toxin, and 10 ng/ml insulin.

3.2.3.2. Cell viability

The MTS reagent's manufacturer's instructions were followed when conducting the cell viability assay. In 96-well plates, 1x106 MCF-7 cells were planted and then incubated for 24 hours. The next day, 5, 10, 20, 30, and 40 uM concentrations of Sulforaphane-Emulsome, Sulforaphane, and Blank Emulsomes were administered into these 96-well microplates. The growth medium was withdrawn after the initial incubation time (24 hours), and then 100 l of the MTT assay was added to the serum-free culture medium. This mixture was then incubated for 4 hours at 37 °C. The samples' under-570nm absorbance was measured using a microplate reader. After 48 hours and 72 hours, the identical operation was carried out. Triplicates of each sample were run on each experiment.

3.2.3.3. Control cell study

MCF10A (Human Breast Endothelial Cell Line) was treated with SLF, SLF-Em, and blank emulsomes in order to determine whether the combination of SF with chemotherapeutic agents is toxic for normal cells. The viability of MCF10A was assessed for each treatment group after 24, 48, and 72 hours using the same technique as described in 3.4.2 by MTT assay.

3.2.3.4. Annexin v – pi apoptosis analysis

Cells from MCF-7 and MCF10A underwent two days of cultivation and treatment, respectively. Different treatment groups were administered to the cells at a concentration of 105 cells/ml, including free SLF, blank emulsomes, and SLF-emulsomes (Both 10uM and 25uM concentrations). The cells were cleaned with PBS and resuspended in the binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl2, pH 7.2). Following that, cells were separated from the medium by adding Trypsin-EDTA (1ml to each plate) and were then incubated for 5 minutes at room temperature. After that, 100ul were transferred to a culture tube and Annexin V-FITC and 5ul of PI dye were added. The tube was then administered the binding buffer, and flow cytometry was performed while adjusting the settings in accordance with the experiment.

CHAPTER 4

4.   RESULTS AND DISCUSSION

4.1.   Sulforaphane's Encapsulation Within the Nano Formulation

The mixture of tripalmitin, cholesterol, DPPC and Sulforaphane in chloroform was used to synthesize the nanoformulation using solvent evaporation technique. It is important to note that sulforaphane was separately dissolved in ethanol and later on added to the chloroform mixture.

The synthesized formulation was proven to be stable during the first five months by the means of the data taken from the zeta potential measurements. Despite that, the formulation that was used for cell culture studies were not older than one month in order to avoid false data that could be influenced by the release of sulforaphane particles from the formulation over the passage of time. Thereby, the nanoformulation was synthesized repeatedly over the course of this study, and encapsulation of sulforaphane was achieved within a range of 0.029 – 0.051 mg/ml (**Table 4.1**).

Table 4.1: Encapsulation amounts of the Sulforaphane-Emulsome nano-formulations.

	Encapsulation (mg/ml)	Molarity (µM)
Production #1	0.035 mg/ml	197 µM
Production #2	0.029 mg/ml	164 µM
Production #3	0.033 mg/ml	186 µM
Production #4	0.051 mg/ml	288 µM

4.2.   Physicochemical Properties of Sulforaphane-Emulsomes

4.2.1.   The mean particle size and zeta potential and polydispersity index

DLS analysis determined the average diameter of distinct SFN-emulsomes as 246.0 nm (**Table 4.2**) - in which the plus-minus sign indicates the margin of average size of numerous formulations made of the same composition. The mean diameter of blank emulsomes was found as 188.3 ± 87.22 nm. SFN-loaded and blank emulsomes had PDI values 0.413 and X, respectively. The zeta potential analysis showed that SFN-loaded emulsomes have in average a less negative zeta potential value (-22.5 mV ± 7.7) than the blank formulation (-31.8 mV ± 6.68) (**Figure 4.1**).

Table 4.2: Comparison of size distribution and zeta Potential between Blank Emulsomes and SFN-emulsomes.

ZetaSizer Sample #1	Size (nm)	Zeta Potential
Blank Emulsomes	188.3 ±	- 31.8 ±
Sulforaphane-Emulsomes	246.0 ±	- 22.5 ±

As demonstrated in **Table 4.3**, the average size of the SFN-Emulsomes varies within a range of 237.1 – 266.8 nm. The size distribution results disclosed that the distributions of the particles are slightly polydisperse (with a moderate PDI value between 0,3 - 0,5).

Table 4.3: Average size, PDI and zeta potential of the nano formulations.

SFN-Em Production	Average size (nm)	Polydispersity Index (PDI)	Zeta Potential (mV)
Production #1	239.2 ±	0.39	- 22.5 ±
Production #2	266.8 ±	0.51	- 26.9 ±
Production #3	241.8 ±	0.40	- 23.2 ±
Production #4	236.1 ±	0.35	- 21.5 ±

a)
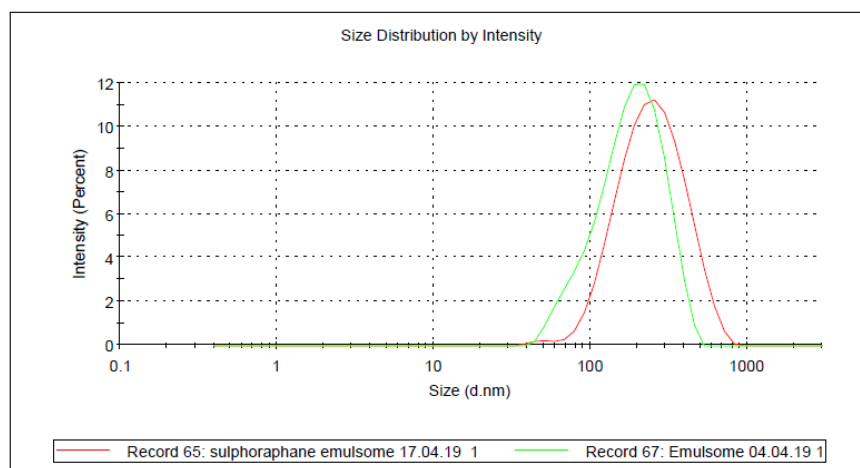
b)
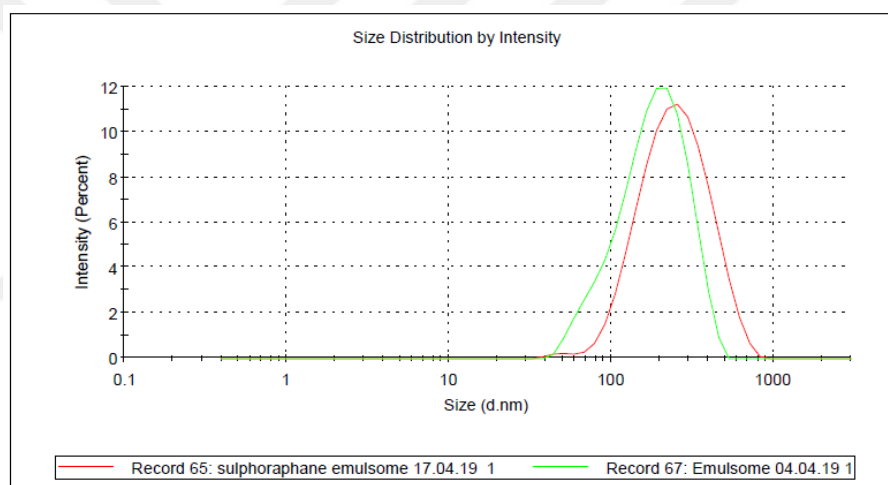
Figure 4.1: Zetasizer results of (a) sulforaphane-emulsomes and (b) blank Emulsomes.

4.2.2. Dispersity in water

Since the mean size of the SFN-loaded emulsomes are found to be within the visibility range of optical microscopy, confocal laser scanning microscopy (LSM 780) was used to visualize the behavior of nanoparticles in aqueous environemtn and provide further evidence for the dispersity characteristics of the formulation. Confocal microscopy images showed that emulsomes are highly dispersed in water and confirmed the DLS data on that aggregate formation does not occur (**Figure 4.2**).
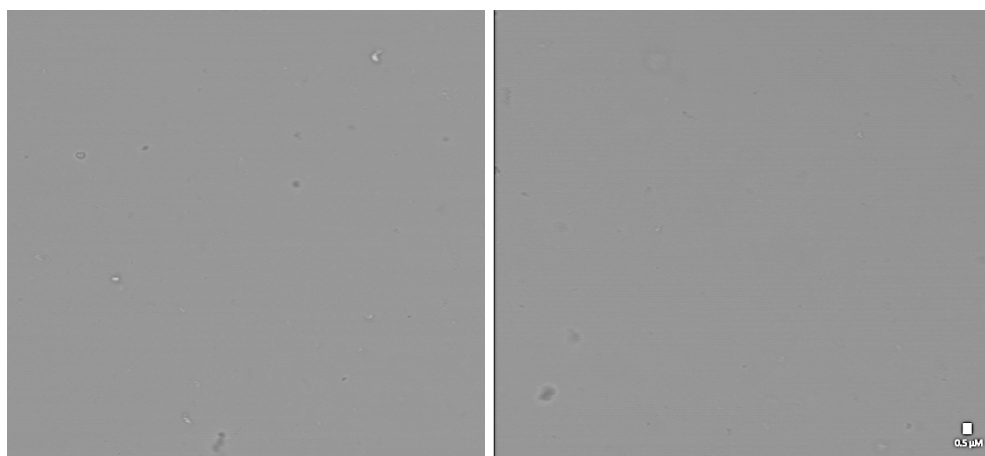
Figure 4.2: Confocal microscopy images of blank and SFN-loaded emulsomes.

4.2.3. Morphology, size and shape of sulforaphane-loaded emulsomes

The size of the SFN-Emulsomes was further observed by scanning electron microscope together with their morphological properties. Based on the image captured at 75.000X magnification, SFN-Emulsomes are spherical in shape, and hence, similar in size and morphology to blank emulsomes **(Figure 4.3)**. The particle size distribution of blank emulsomes appear largely vary between 100 and 200 nm, SFN-loaded emulsomes have sizes mostly within the range between 200 and 300 nm, thereby exhibiting a uniform size and shape characteristics.
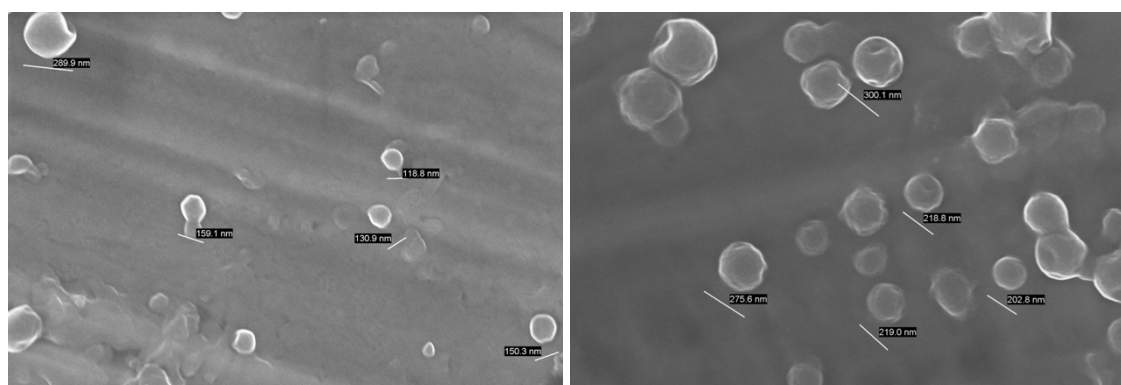
Figure 4.3: SEM images of blank and SFN-loaded emulsomes.

4.2.4. Quantification of sulforaphane's emulsome

Two methods including HPLC, and absorbance assay were used to quantify SFN and calculate its encapsulation within the emulsomes. Firstly as the more sensitive approach, HPLC analysis were carried out to detect and quantify SFN encapsulated within the emulsomes. For SFN-loaded emulsomes, a peak was observed at 7.5 minutes that is consistent with the peak obtained when SFN was analyzed separately as the standard

47

(**Figure 4.4**). Based on this area under the peaks, the corresponding concentrations of SFN inside emulsomes were calculated as presented in **Table 4.4**.
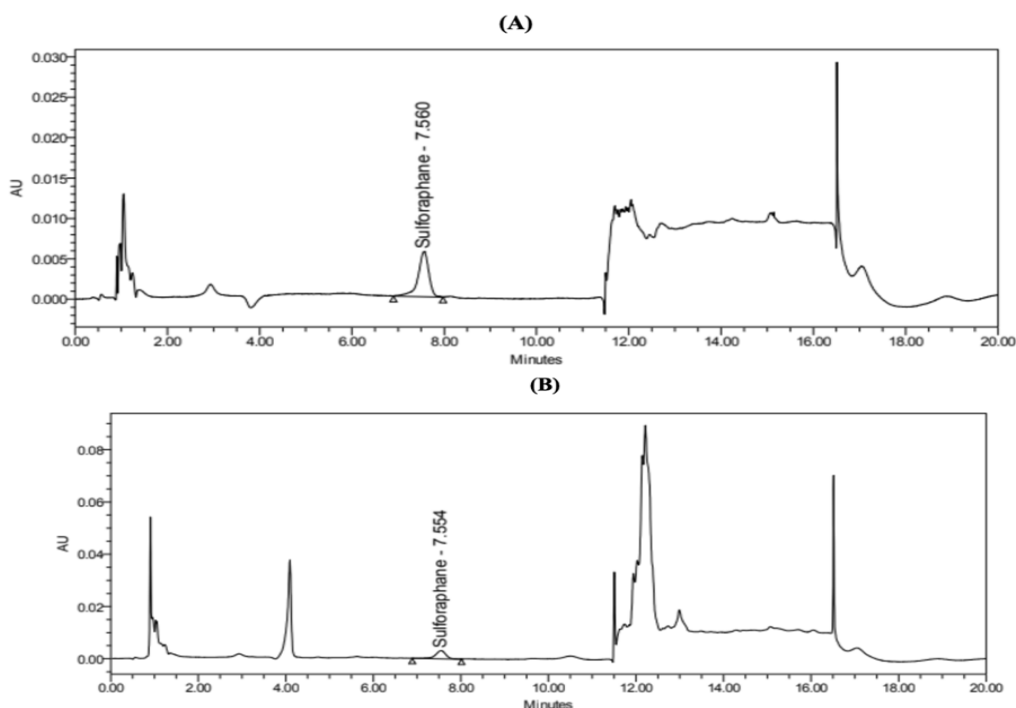
Figure 4.4: Compositional analysis of sulforaphane and sulforaphane-emulsomes. The HPLC technique is used to quantify the amount of Sulforaphane encapsulated inside the Emulsome.

Table 4.4: HPLC data used for quantification of sulforaphane within the emulsome.

Production #1 & #3		Area	Average Area	Concentration (mg/ml)
	Standard 1	118160	118358,5	0,050
	Standard 2	118557		
1:4 dilution	SLF-Em 1	18157	19601,5	0,036
	SLF-Em 1	21046		
1:4 dilution	SLF-Em 2	21462	19533,5	0,033
	SLF-Em 2	17605		

As an alternative quantification methodology, absorbance assay was used to determine if concentration of SFN in emulsomes can be determined with this simpler methodology with the same certainty compared to HPLC. A calibration curve for SFN was optimized and thereafter, the drug content of SFN was measured using UV-Vis spectrophotometer (Thermo Fisher Scientific). The result obtained through this method matched with the

values previously acquired by HPLC, thereby provided an alternative easier methodology for quantification of SFN within the emulsomes (**Table 4.4**).

4.2.5. Cell viability analysis of mcf-7 (MTT Assay)

MTT assay was performed in order to study the combined effects of chemo-preventive drug SFNs on the MCF-7 cell lines. SFN was used in its unmodified form at the following concentrations: 1,5,10,25,50,100 µM and the average of the three analysis was performed and demonstrated as shown in (**Figure 4.5**). The free SFN was able to reduce the cell viability by an average factor of 97% but it was observed that as the concentration increased, the less effect Sulforaphane had on the cells. On the other hand, when SFN-Em was administrated, as the concentration increased from 1 to 100 µM, the cell viability drastically decreased from 87% to 39%, respectively (**Figure 4.8**).

Figure 4.5: Cell Viability results after the passage of 24 hours for Free Sulforaphane, Sulforaphane-Emulsomes and Blank Emulsomes.

This type of progress was observed after the passage of 48 hours, where the cell viability of free Sulforaphane fluctuated between 50% and 15% as the concentration increased from 1uM to 100uM. As for Sulforaphane-Emulsomes, the cell viability decreased gradually from 77% to 27% when 5µM and 100µM were administrated, respectively. In correspondence to Blank-Emulsomes, the cell viability was observed to have decreased as well as the concentration increased; For a concentration of 1µM, the cell viability was approximately 99%, but as the dosage was amplified (100µM), the cell viability reached around 57% (**Figure 4.6**).

Figure 4.6: Cell Viability results after the passage of 48 hours for Free Sulforaphane, Sulforaphane-Emulsomes and Blank Emulsomes.

After the passage of 72 hours, free sulforaphane operated in a similar manner and caused an 85% reduction in cell viability when a concentration of 100μM was administrated. Following Sulforaphane Emulsomes, the cell viability at each concentration was further reduced by a factor of 10% when compared with the data presented after 24 hours and 48 hours. Whereas, no considerable changes have been detected for Blank emulsomes, in which the cells exhibited a parallel behavior after the passage of 72 hours as they did after the first 48 hours.

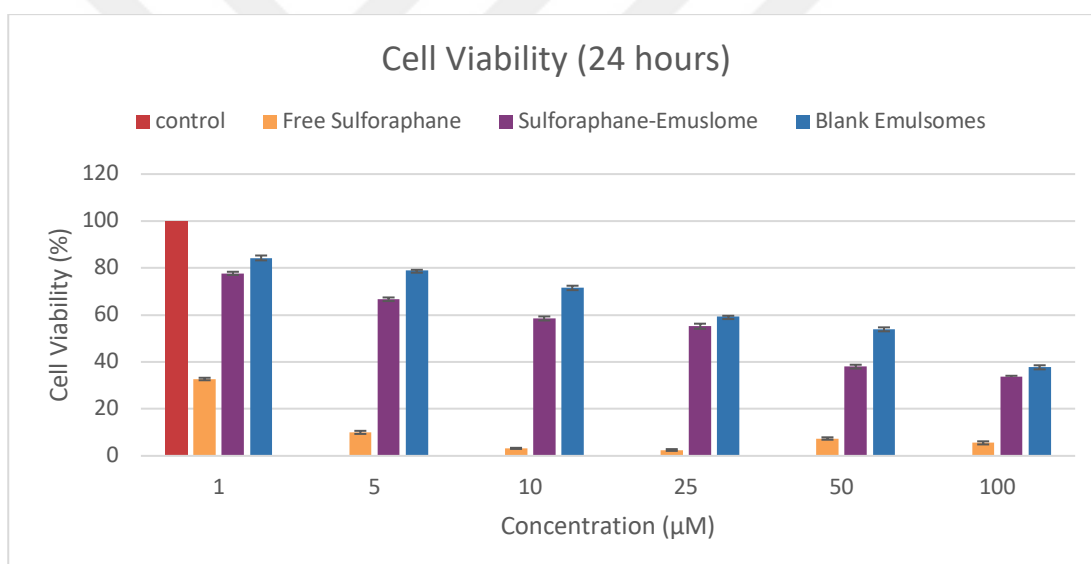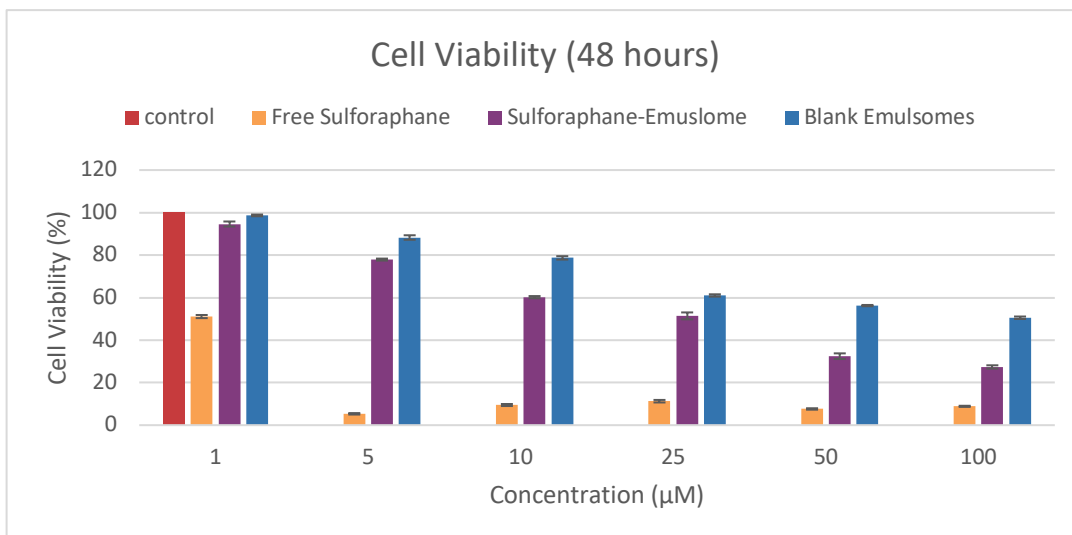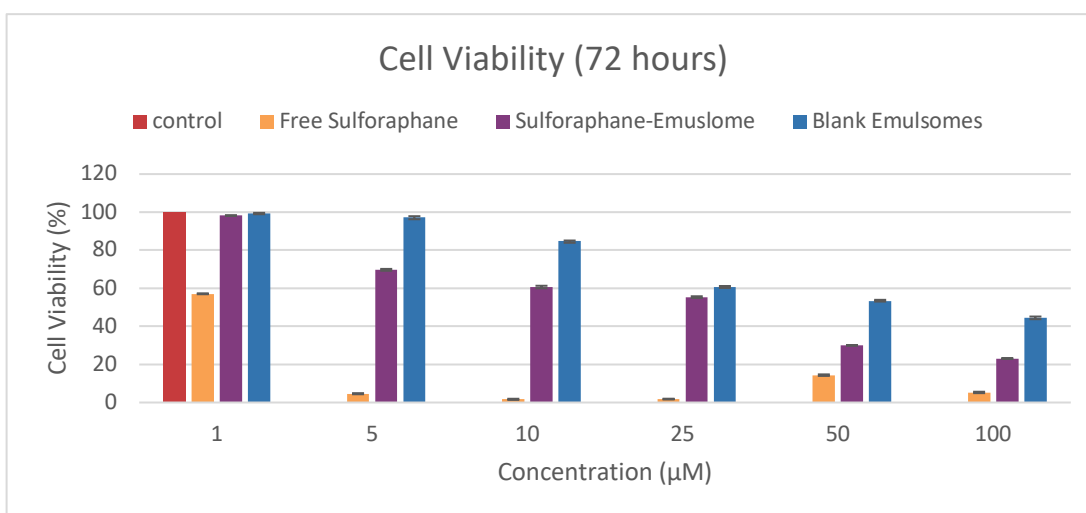**Figure 4.7:** Cell Viability results after the passage of 72 hours for Free Sulforaphane, Sulforaphane-Emulsomes and Blank Emulsomes.

When the analysis of blank emulsomes alone is taken into consideration, it is important to notice that the carrier exhibited a fellow reduction in the cell viability that reached around 41% when 100µM was administrated, and the amount fluctuated slightly between 45 and 42% after the passage of 48hrs and 72hrs, correspondingly. Still, at concentrations of 10uM and 25µM, the cell viability moderately stretched between 80 and 60%. Whereas Blank Emulsomes demonstrated a little to no faint reduction in cell viability that ranged between 100 and 90% for the lower concentrations of 1µM and 5 µM, respectively.
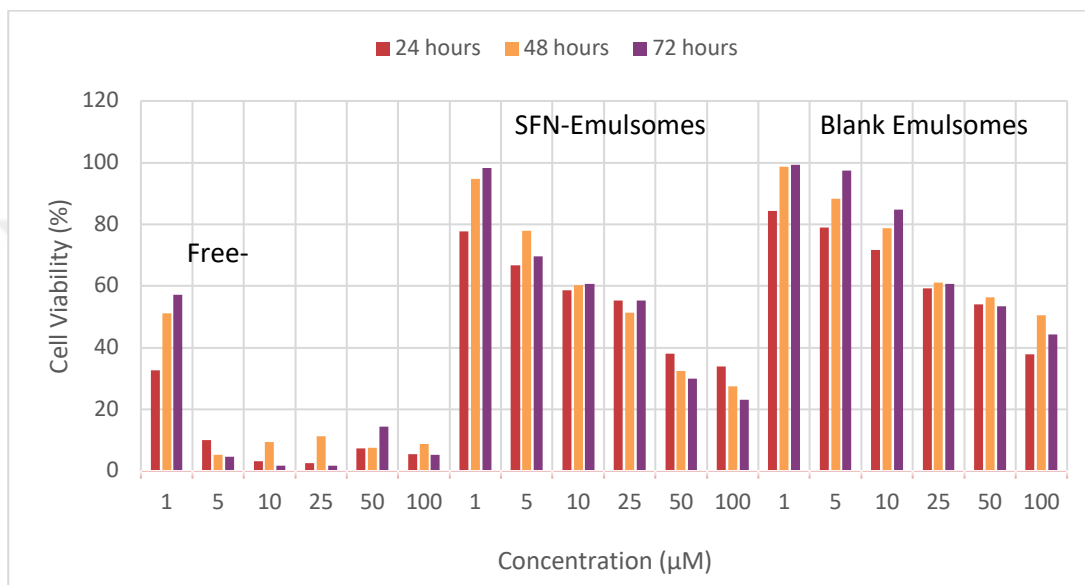
Figure 4.8: Cell Viability analysis of Sulforaphane, Sulforaphane-Emulsomes, Blank Emulsomes after the passage of 24 hours, 48 hours and 72 hours.

4.2.6. IC$_{50}$ value calculations

IC50 value was calculated for both free SFN and SFN-Emulsomes based on the cell viability results obtained previously after the passage of 24, 48, and 72 hours using (Prism 9 software). Based on the graph attained for average log concentration versus cell viability, the IC$_{50}$ value of free SFN was deducted to be an average of (0.4 µM for 24 hours, 0.9 µM for 48 hours and 1.155 µM for 72 hours) as shown in (**Figure 4.8**). Whereas for SFN-Emulsomes, the IC$_{50}$ value was shown to be (23.4 µM for 24 hours, 22.9 µM for 48 hours and 21.11 µM for 72 hours) as shown in (. The following outcomes further assisted by highlighting the effect of these concentration for the control group study and apoptosis analysis.
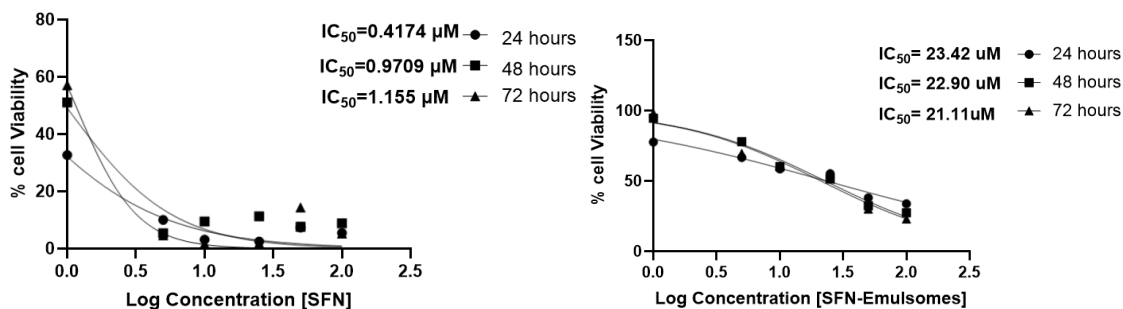
Figure 4.9: IC50 values for free Sulforaphane and Sulforaphane Emulsomes after the passage of 24 hours, 48 hours, 72 hours.

4.2.7. Cell viability analysis of mcf10-A (MTT Assay)

In order to study the effect of SFN-Emulsomes over the control group comprising of MCF10A cell lines, an MTT assay was performed thrice, following the same procedure used on the cancerous cells in order to unify the results and make them comparable. The average of the thrice study was gathered and presented below (**Figure 4.10**).
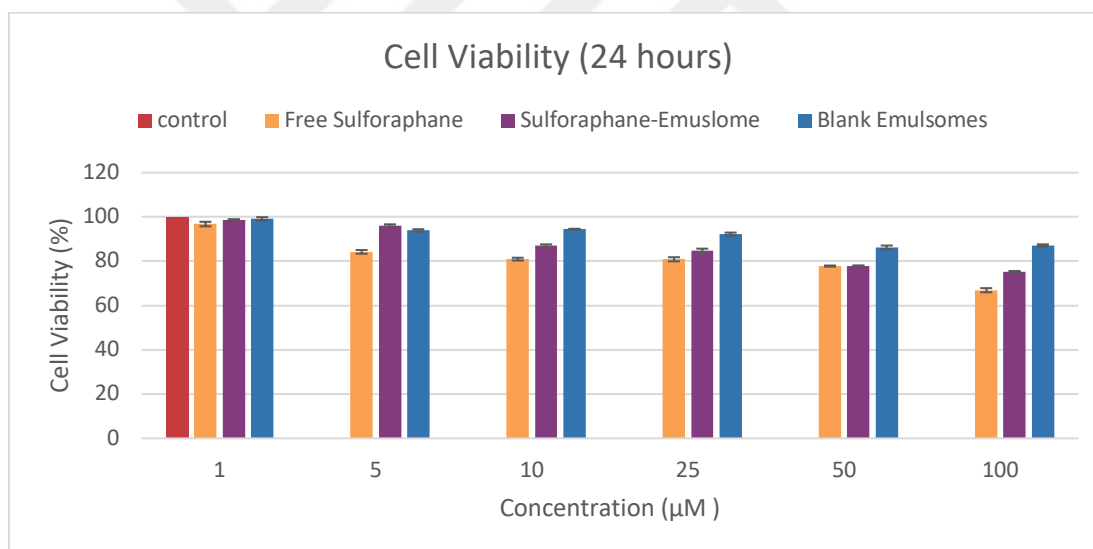
Figure 4.10: Cell Viability percentage of MCF10-A after the passage of 24 hours for Free Sulforaphane, Sulforaphane-Emulsomes and Blank-Emulsomes.

The mentioned controlled study was carried out in order to prove that the drug formulation is not harmful to healthy epithelial cells and is mostly toxic against cancerous ones. The same steps were followed by testing free Sulforaphane, SFN-Emulsomes, and blank emulsomes on MCF10A cells. The result obtained at the first 24 hours proved the suggested theory right.

Free Sulforaphane's effect on the cell viability didn't exceed 30% at a high concentration of 100 µM. Whereas the cell viability when SFN-Em was administrated ranged between 80 – 90%, and the blank Emulsome contributed to an average of 10 – 5% decrease in the healthy cell's viability.

After the passage of 48 hours, free-sulforaphane registered a similar result in which the cell viability was reduced from 98% into 70 % as the dosage increased from 1 to 100 µM. In reference to the synthesized nano-formulation, SFN-Emulsomes exhibited a parallel behavior for the concentration of 1, 5, 10 µM. Only for the concentrations of 25, 50 and 100 µM, the nano-formulation displayed a sturdier performance in which the cell viability was decreased by a factor of 22, 30, and 40%, respectively (**Figure 4.11**).
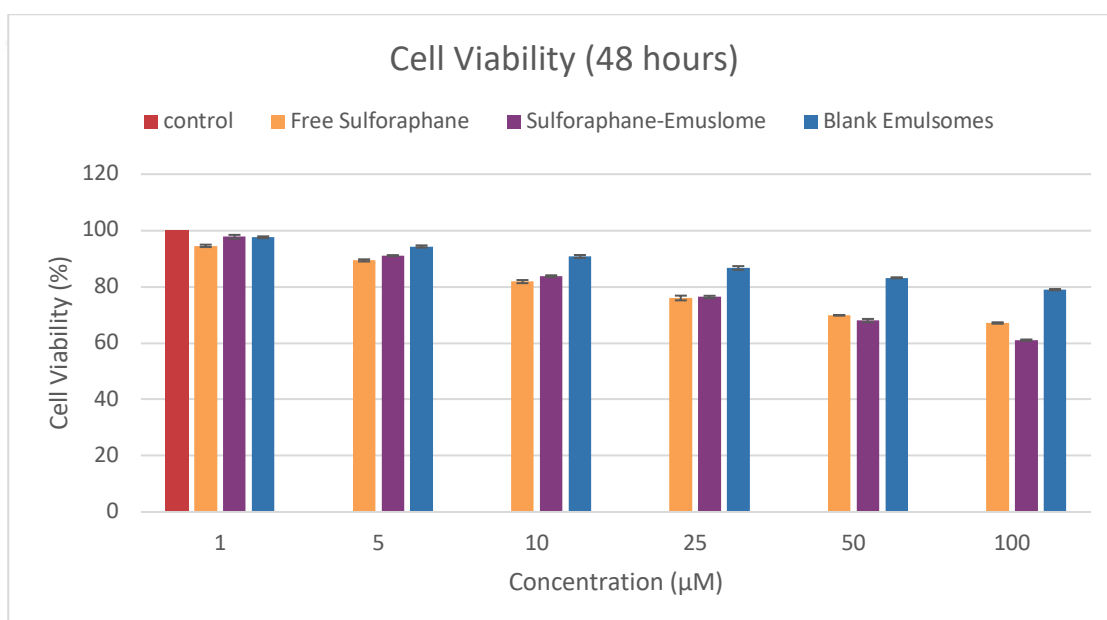
Figure 4.11: Cell Viability percentage of MCF10-A after the passage of 48 hours for Free Sulforaphane, Sulforaphane-Emulsomes and Blank-Emulsomes.

The results gathered after the passage of 72 hours were analogous to those obtained after 48 hours in which no considerable alteration was observed for Sulforaphane-Emulsomes and Blank Emulsomes. In which, SFN-Em conserved the cell viability between values of (95% and 60%). On the other hand, free Sulforaphane's effect was higher when the 100 uM was administrated, in which the cell viability was diminished by a factor of 40% (**Figure 4.12**).
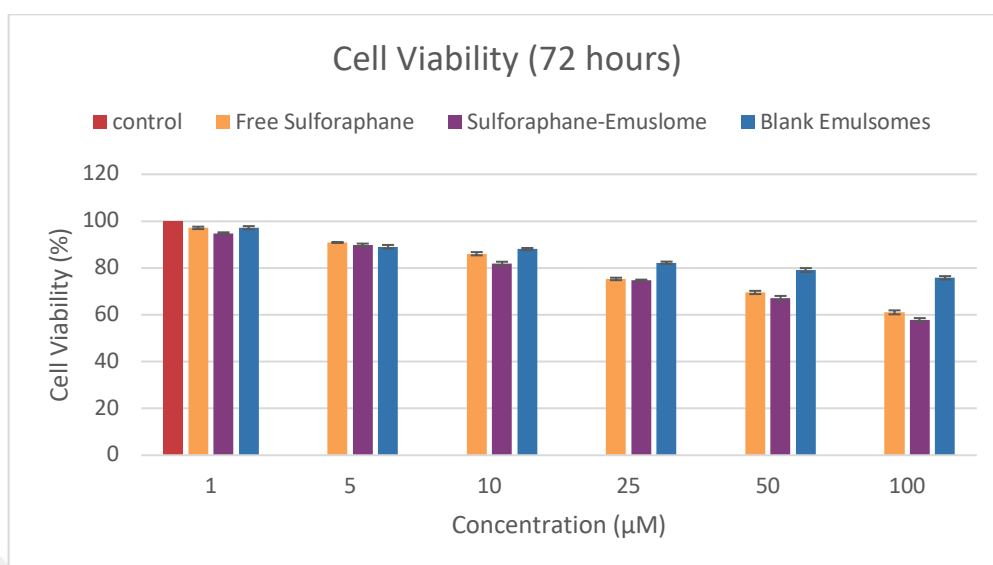
Figure 4.12: Cell Viability percentage of MCF10-A after the passage of 72 hours for Free Sulforaphane, Sulforaphane-Emulsomes and Blank-Emulsomes.
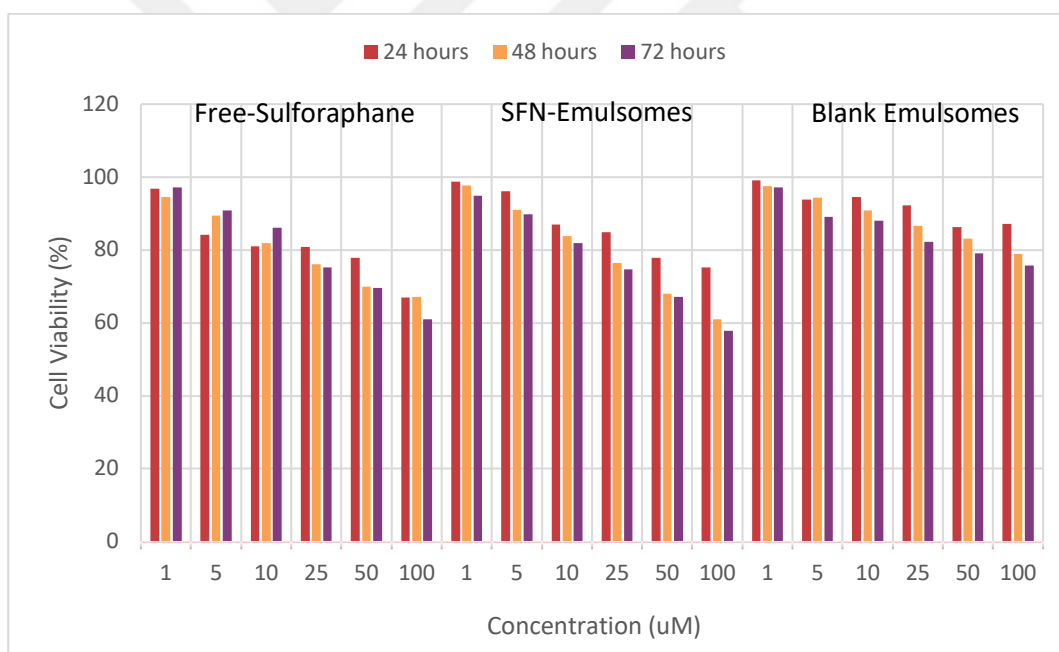

Figure 4.13: Cell Viability analysis of MCF10-A after the administration of Sulforaphane, Sulforaphane-Emulsomes, Blank Emulsomes following the passage of 24 hours, 48 hours and 72 hours.

4.2.8. Annexin v – pi apoptosis analysis for mcf-7

Following the cell viability that was tested for both cancerous and healthy cells, the next step was required to check the apoptosis mechanism of SFN on both cell lines. Flow

cytometry was carried out by the method mentioned in part 3.4.4 and measured after the passage of 24 hours and 48 hours (**Figure 4.14**).

a)

b)
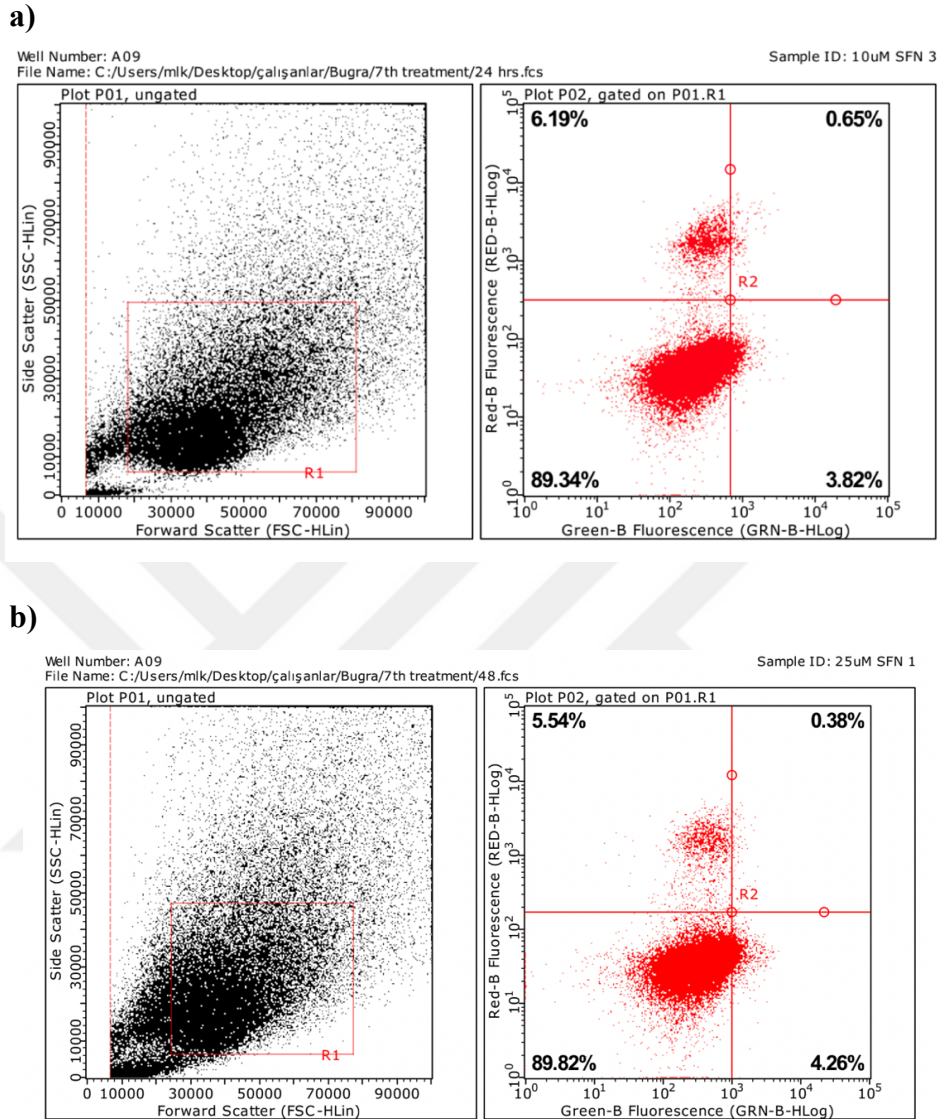
Figure 4.14: Flow cytometry results of MCF-7 after the passage of 24 hours of **(a)** 10uM Free SFN and **(b)** 25uM Free SFN.

When SFN-Emulsomes was administrated, the apoptosis reached 15% – 24.6 % for the corresponding concentrations of 10uM – 25uM. As for the late apoptosis, the values ranged between 2 – 3%, respectively (**Figure 4.15**).

(a)
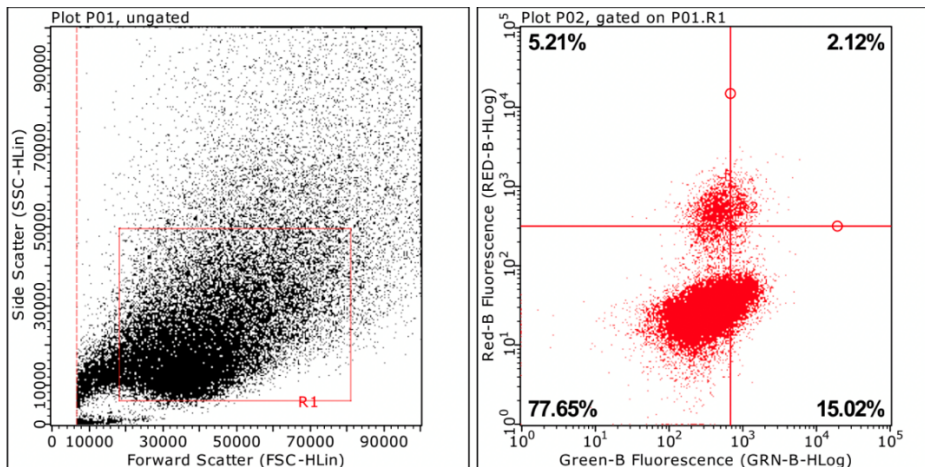
(b)
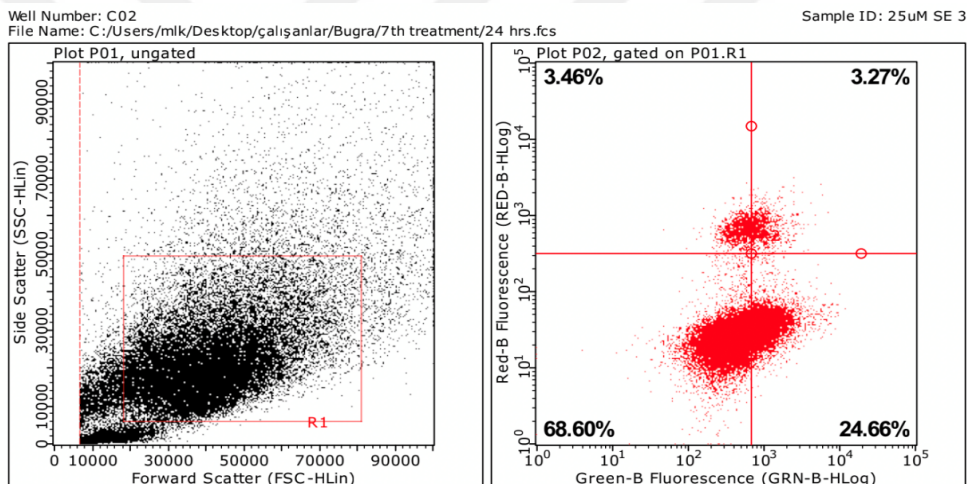
Figure 4.15: Flow cytometry results of MCF-7 after the passage of 24 hours of **(a)** 10uM SFN-Emulsomes and **(b)** 25uM SFN-Emulsomes.

Following these results, Blank Emulsomes' effect didn't exceed 2.7% for the 25uM concentration and after the passage of 24 hours, and had a faint to no effect, exhibiting a 0.8% early apoptosis for the 10uM concentration. In reference to the late apoptosis, the percentage didn't cross 0.28% for both concentrations (**Figure 4.16**).

(a)

(b)
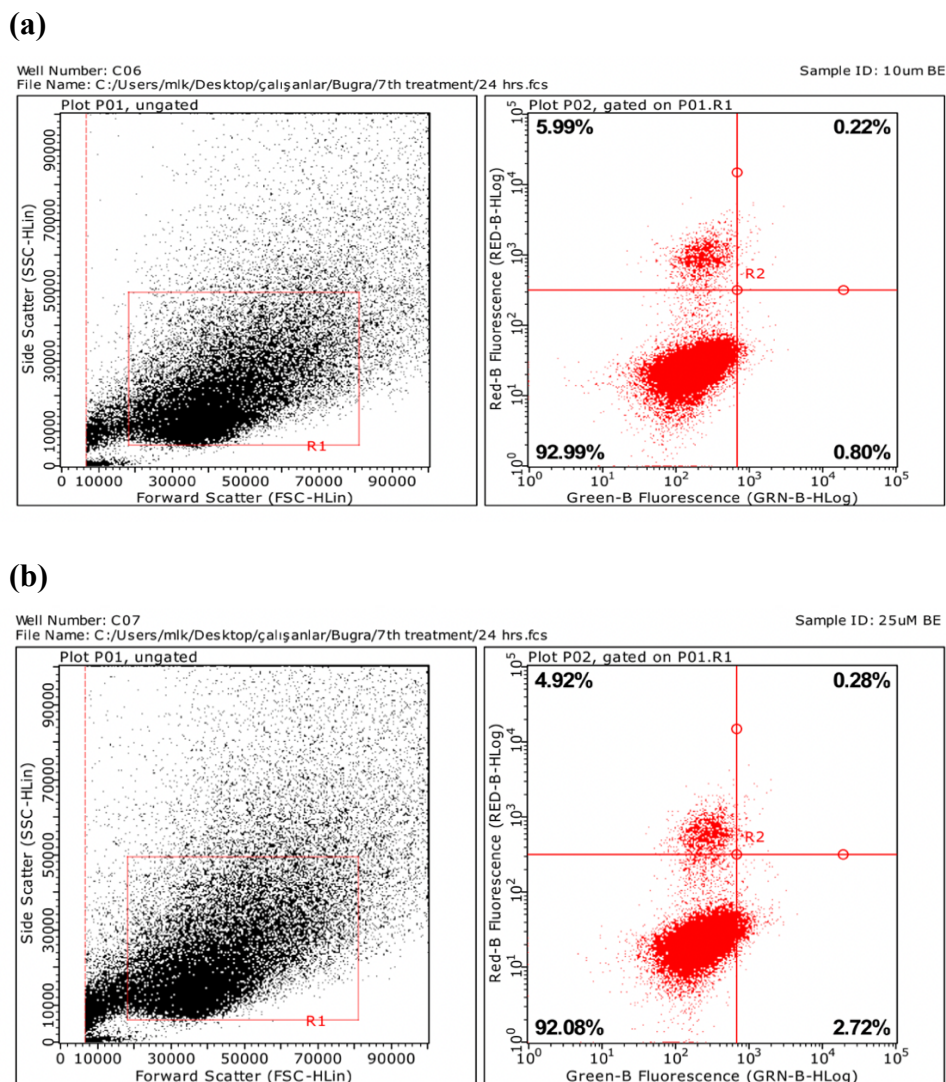
Figure 4.16: Flow cytometry results of MCF-7 after the passage of 24 hours of **(a)** 10uM Blank Emulsomes and **(b)** 25uM Blank Emulsomes.
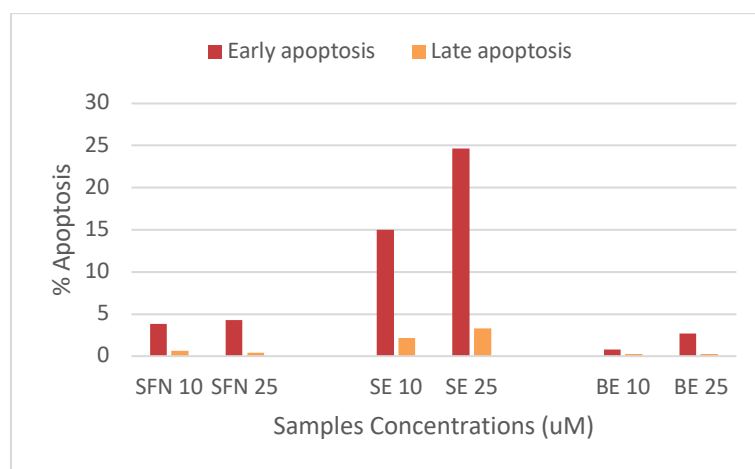
Figure 4.17: Cytometry results illustrate the percentages of early and late apoptotic cells after 24 hours between Free SFN, SFN-Emulsomes (SE) and Blank Emulsomes (BE).

Following the passage of 48 hours, the early apoptosis detected for Free Sulforaphane differed slightly from the data demonstrated above, in which it exhibited a lower percentage of 0.86% for 10uM. However, a similar factor of 4.26% for the 25uM dosage was reported (**Figure 4.18**).

(a)

(b)
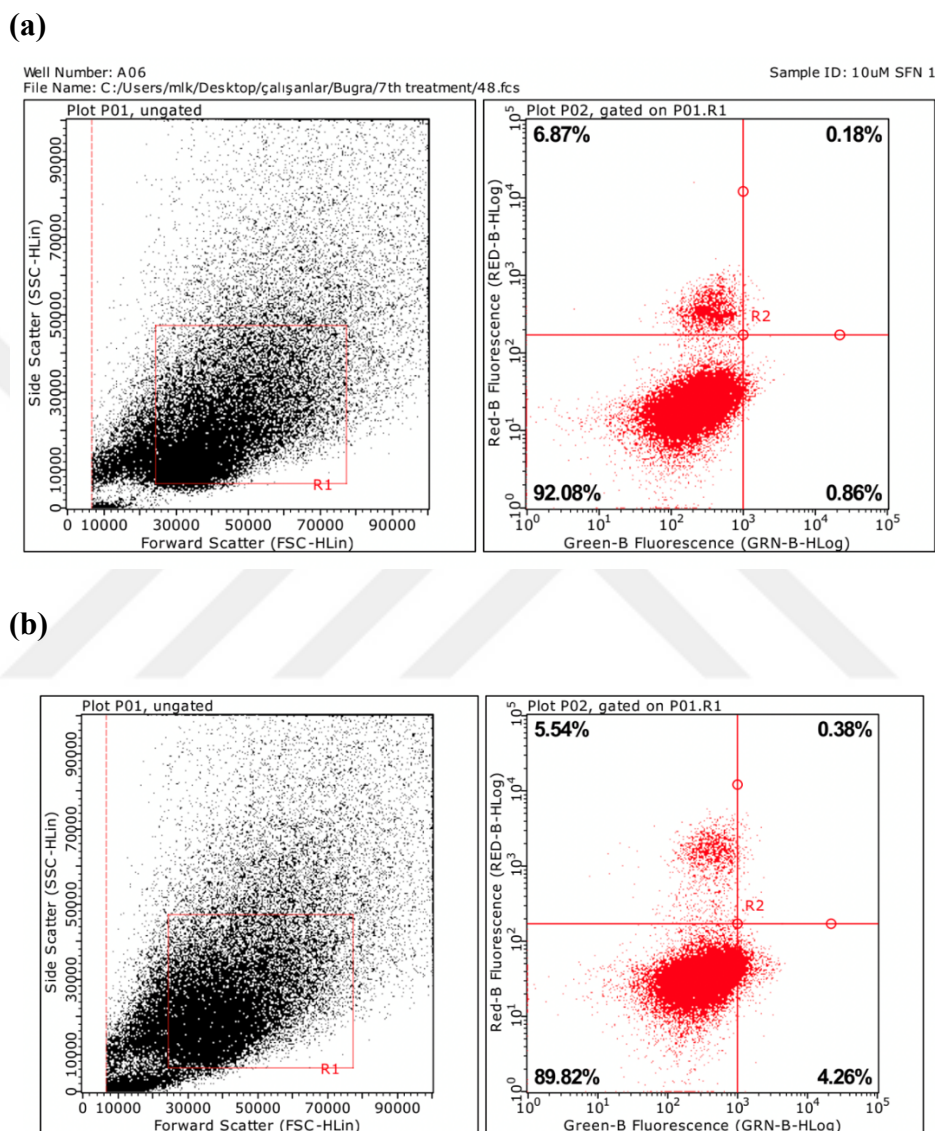
Figure 4.18: Flow cytometry results of MCF-7 after the passage of 48 hours of **(a)** 10uM Free SFN and **(b)** 25uM Free SFN.

In correspondence to the nano-formulation SFN-Emulsome, the early apoptosis outcomes specified an assessment ranging between 8.5 – 27.9% for the same concentrations of 10 and 25uM, correspondingly. In addition, the late apoptosis values were similar and in agreement after 48 hours as same as those found within 24 hours. It is also noticed that

the necrotic values had increased slightly, in which it reached 7.4% for the 10uM SFN-Em concentration.

(a)

(b)
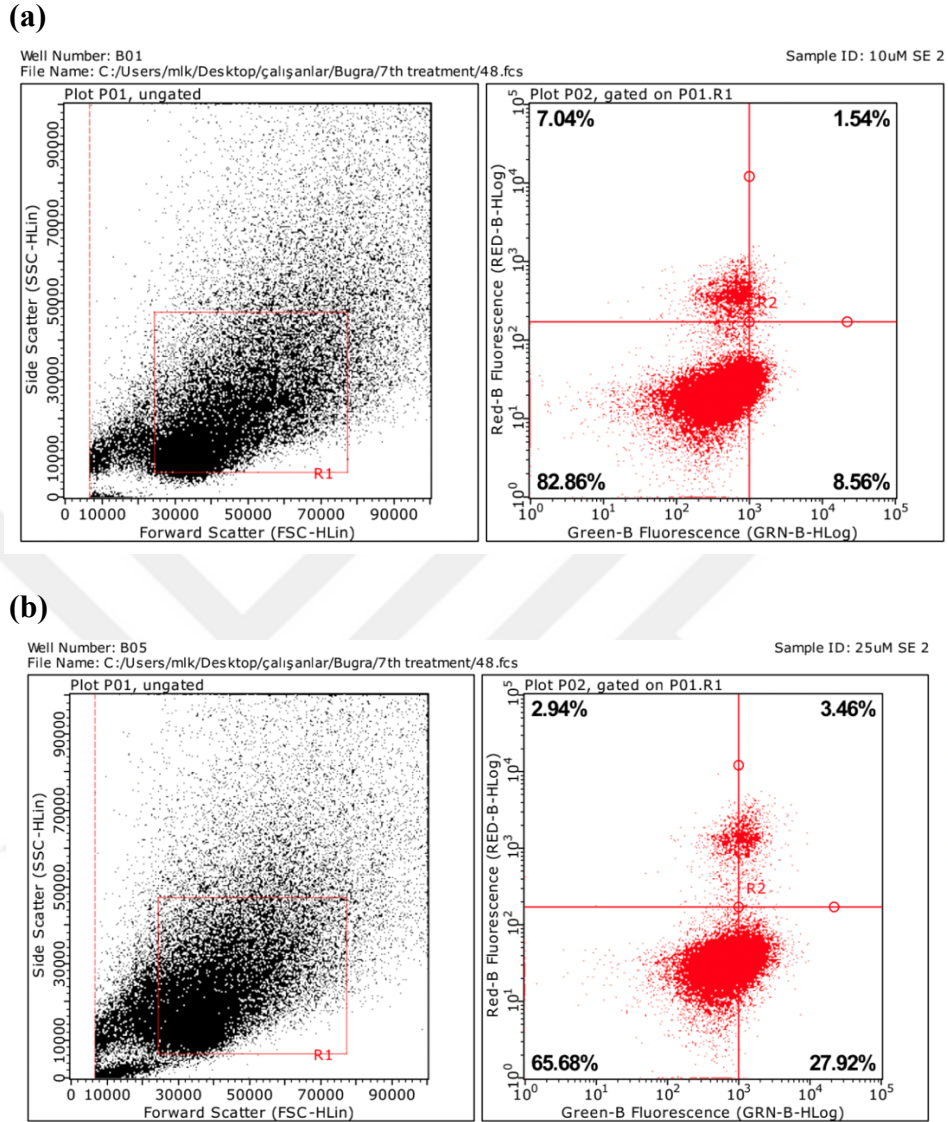
Figure 4.19: Flow cytometry results of MCF-7 after the passage of 48 hours of **(a)** 10uM SFN-Emulsomes and **(b)** 25uM SFN-Emulsomes.

Furthermore, Blank Emulsomes expressed a considerable increase in the early apoptosis percentages for both concentrations. In which, 4.2% and 7.11% were testified for 10uM and 25uM concentrations, with a late apoptosis slightly higher than that after the course of 24 hours, in which it reached approximately 1% for the 25uM dosage (**Figure 4.20**).

(a)
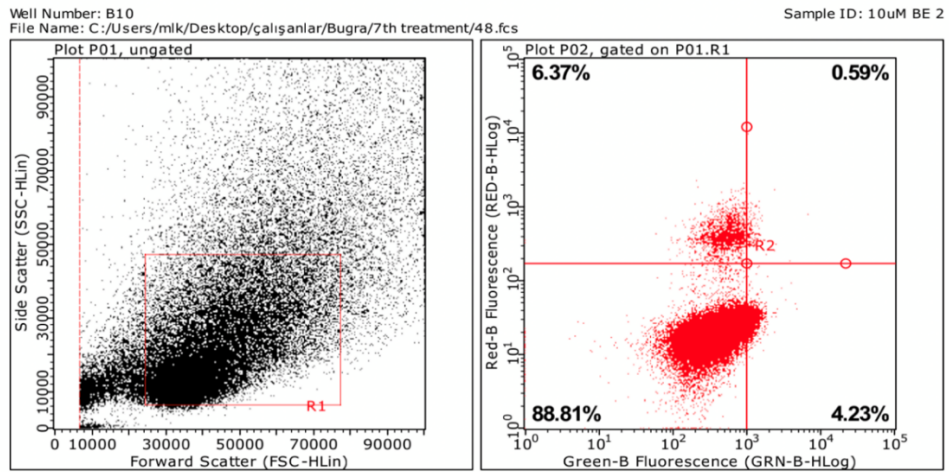
(b)
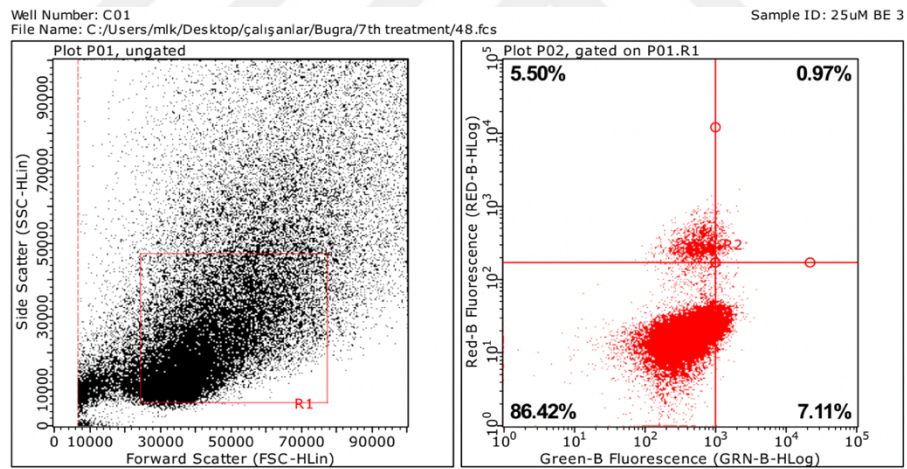
Figure 4.20: Flow cytometry results of MCF-7 after the passage of 48 hours of **(a)** 10uM Blank Emulsomes and **(b)** 25uM Blank Emulsomes.
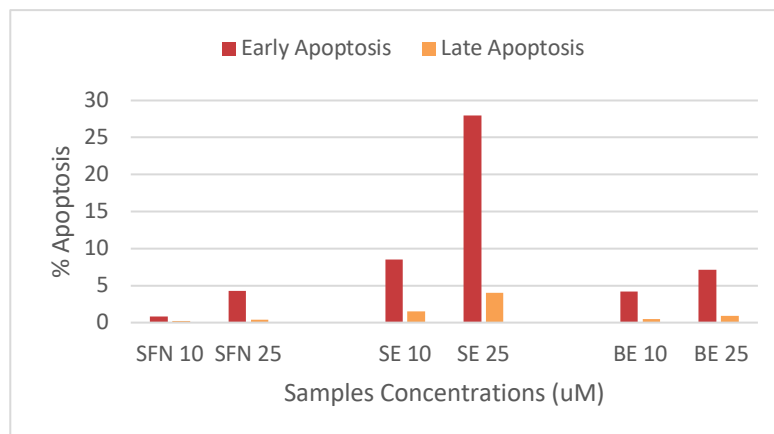
Figure 4.21: Cytometry results illustrate the percentages of early and late apoptotic cells after 48 hours between Free SFN, SFN-Emulsomes (SE) and Blank Emulsomes (BE).

4.2.9. Annexin v – pi apoptosis analysis for mcf10-A

Following the apoptosis analysis carried on the cancerous cells, it was essential to carry out the same procedure on the control cell line group MCF10-A in order to certify the previously obtained data and clarify that the nano-formulation exhibits a negligible toxicity over healthy epithelial cells. Both 10uM and 25uM of free sulforaphane exhibited 0% early apoptosis percentages with inconsiderable late apoptosis values (**Figure 4.22**).
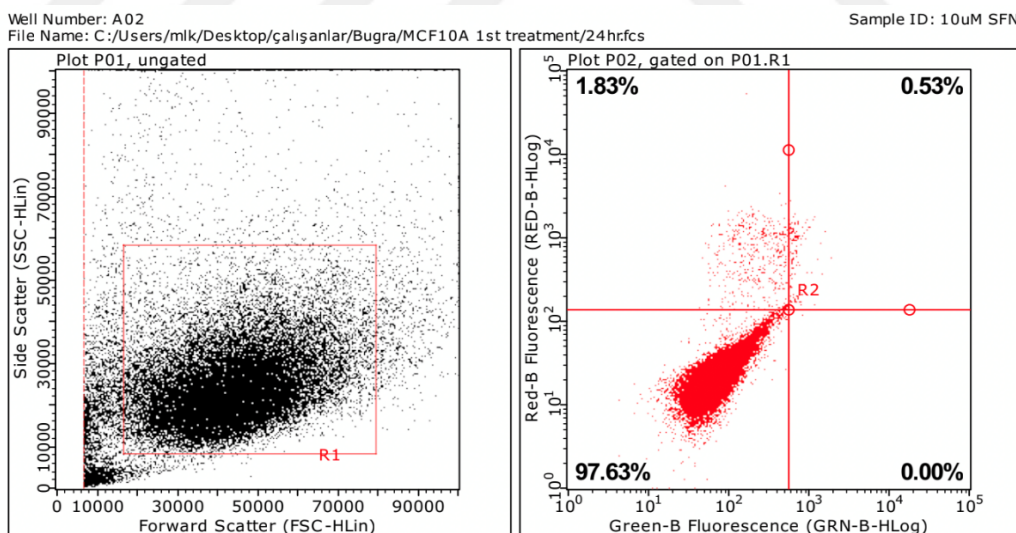
Figure 4.22: Flow cytometry results of MCF10-A after the passage of 24 hours of 10uM Free SFN.
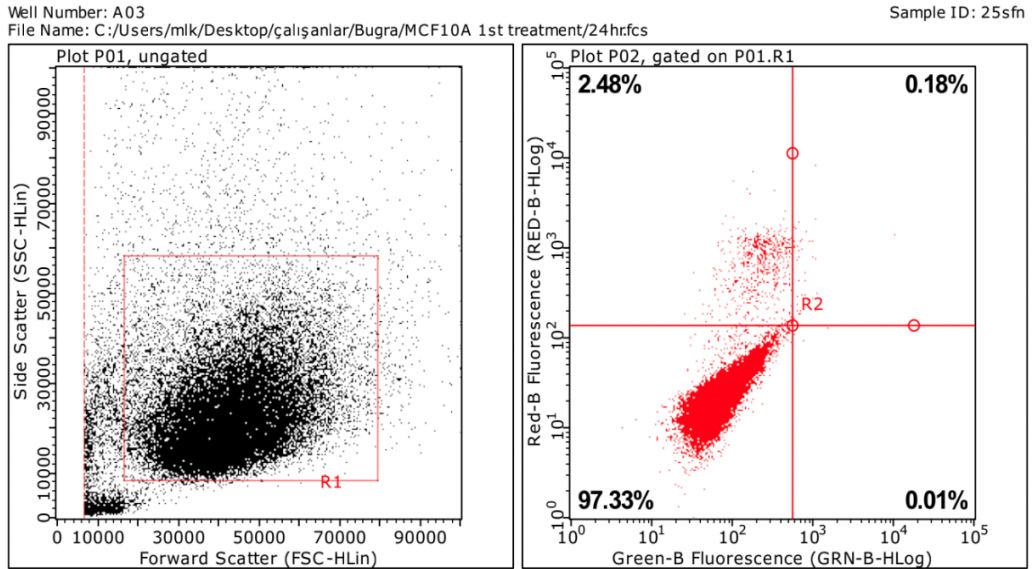
Figure 4.23: Flow cytometry results of MCF10-A after the passage of 24 hours of 25uM Free SFN.

In agreement with free Sulforaphane, both SFN-Emulsomes and blank Emulsomes barely demonstrated any effect on the MCF10-A cell lines for both concentrations of 10uM and 25uM. In which the early apoptosis assessment didn't cross 1% for the drug carrier and the blank Emulsomes mutually (**Figure 4.24** and **Figure 4.25**).
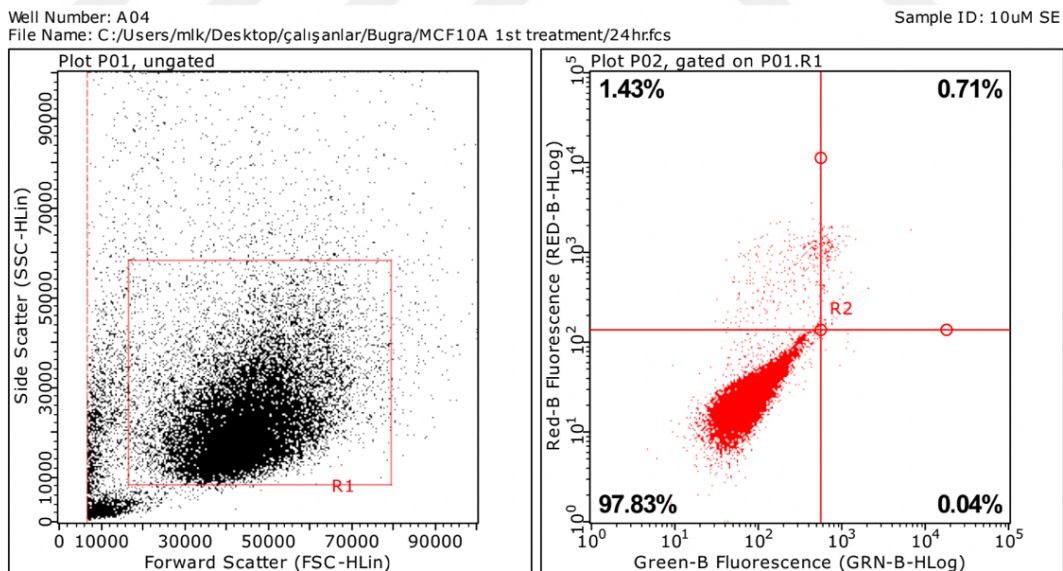
Figure 4.24: Flow cytometry results of MCF10-A after the passage of 24 hours of 10uM SFN-Emulsomes
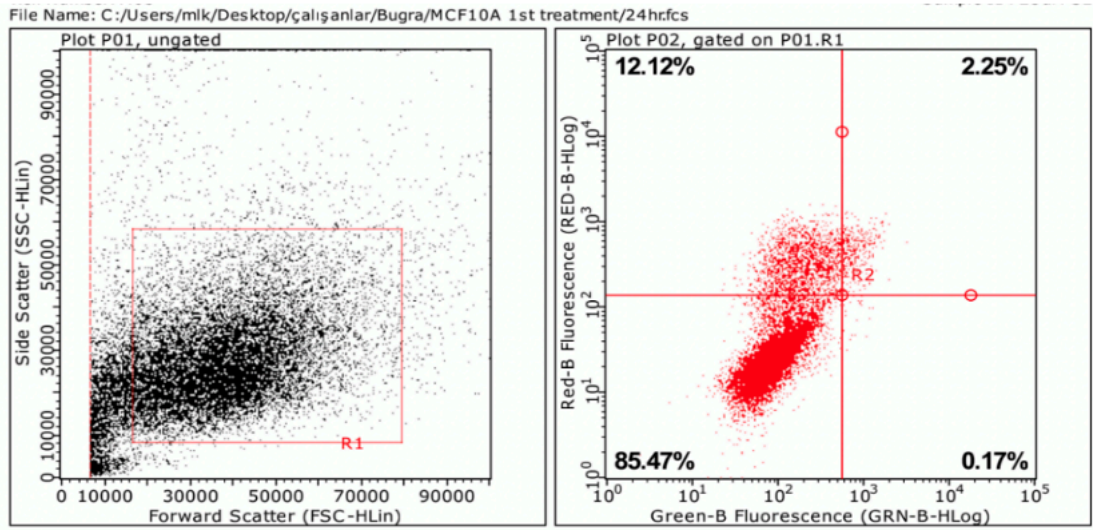
Figure 4.25: Flow cytometry results of MCF10-A after the passage of 24 hours of 25uM SFN-Emulsomes.

4.3. Discussion

It has been reported that the intake of cruciferous vegetables that contain Sulforaphane is effective for a remarkable reduction in the tumor progression. Although sulforaphane can also be obtained by eating cruciferous vegetables such as Brussel sprouts, broccoli, cauliflower, turnip, radish, watercress and cabbage, it is washed out of the body in large amounts due to its low bioavailability[60]. To overcome this problem, this study aimed to encapsulate Sulforaphane within emulsomes in order to enable its delivery to the targeted cell with certain amounts assuring a therapeutic effect.

In this study, Sulforaphane loaded Emulsome nanoparticles were used as vehicles to investigate the anti-cancer effect of the Sulforaphane on breast cancer cell within this lipid-based formulation. Since the very low solubility of the Sulforaphane limits its medicinal applications, nanocarrier drug delivery systems counter for a prominent approach to enhance and improve the novel, diversly characterized SFN in cancer protection. Encapsulation of SFN inside the emulsomes noticeably stipulated an improvement in its solubility. Herein, the characteristics of the SFN-Em were analyzed furthermore and then its effect on cell viability in both MCF-7 tumor cell lines and MCF10A healthy line was evaluated, in addition to apoptosis tests to further affirm Sulforaphane's anti-cancer properties.

Sulforaphane was successfully loaded (0.033 mg/ml) within the Emulsome as indicated by the HPLC and UV absorption data (**Figure 4.4** and **Table 4.4**) The graphs obtained through HPLC showed a consistent peak of Sulforaphane-Emulsome when compared with the standard Sulforaphane graph. In order to question whether the Sulforaphane's encapsulation had an effect over the size and potential of the Emulsome, ZetaSizer analysis was used. We observed a slight shift in the average size (188 nm to 239 nm) - this range falls into the desired size (200 nm), which is consistent with our target. The constancy of the nanoparticle is related to the electron charge on its outermost shell. The core that is composed of lipid and fatty acids is responsible for the negative effect. The same shift in the negative charge was observed in another article "Thakkar A et al., 2016" [98] where they loaded SLF into a solid-lipid nanoparticle which proves the reliability of our results.

In Soni et al,.2018, a Sulforaphane decorated nanoparticle was analyzed for its anti-cancer properties, the size of the encapsulated particles demonstrated a value of 147.23± 5.321,

which conveniently falls into our range. In addition to that, the Zeta potential of the SFN loaded nanoparticles were observed to be -23.5 ± 2.45 mV which satisfies the findings we got through our four productions, which fluctuated between (– 21mV and – 26 mV). The zeta potential signifies the constancy and stability of the distributed particles in the dispersion medium. When the value is high, it signals the higher repulsion force between particles, thereby diminishing the possibility of aggregation. It is essential to note that the negative value of the zeta potential shows that the adjusted SFN-Emulsomes had acquired a decent stability profile and dispersion attribute [96].

Furthermore, in drug delivery applications exploiting lipid-based carriers, such as liposome and emulsomes formulations, a PDI of 0.3 and below is contemplated to be adequate and implies a homogenous population of phospholipid vesicles [99]. The PDI findings through our particle characterizations validated the literature in which the values ranged along the 0.3 boundaries. Another study done through encapsulating Sulforaphane into a loaded nanostructured lipid carrier (Soni et al,. 2017) optimized the size distribution and potential. Thereby, the outcomes agreed with our practical findings, in which the size of the nano formulation indicated a value of 145.38 nm, and a zeta potential of −25.12 mV.

Moreover, no substantial difference was perceived in sizes of blank emulsomes and SFN-Emulsomes. It was obvious that the zeta potential values of SLF-Emulsomes were more negatively charged which is accredited to the existence of a negatively charged molecule, which is Sulforaphane, within the formulation. This negative charge could be addressed as an advantage rather a limitation as it aids in stabilizing the compound.

Along with the observed images of SEM (**Figure 4.3**), it is clear that the synthesized SFN-Em's size fall into the 200 nm range and is characterized with its spherical morphology. In SEM, dried samples were used for the characterization analysis, that's why the test of the samples again by using Confocal Laser Scanning Microscopy was needed in order to visualize the dispersity of the particles and investigate its colloidal behavior in a solution. Based on that, no significant aggregation was detected (**Figure 4.2**) confirming the size distribution data and the PDI obtained by ZetaSizer (**Table 4.3**).

The in vitro study on effects of SFN-Emulsomes in the case of MCF-7 cells was performed using MTT assay (**Figure 4.8**). Analysis of the MTT data exhibited that SFN-Emulsomes at all concentrations significantly reduced cell viability compared with Blank

Emulsomes. Totally 1 mg/ml of free SF was used in this experiment and diluted into the assigned concentrations accordingly. Moreover, it was repeated in same concentrations of SFN-Emulsomes and blank emulsomes in addition to the cultured cells without any treatment as control group. The MTT assay indicated that SFN directly inhibited MCF-7 cell growth in vitro (The cell viability percentages ranged between 30 – 8 %).

The MTT assay exhibited that SFN-loaded Emulsomes directly inhibited MCF-7 cell growth in vitro at lower concentrations when compared to the literature (The cell viability percentages ranged between 78 – 38 % as observed in (**Figure 4.6**). To prove that, when 5 µM concentration was administrated, the cell Viability reached 70% at the first 24 hours, which was lower and more effective than the finding of Ko et al., 2013, in which they obtained a 98 % cell viability for the same concentration when they used PLGA microspheres as an encapsulation technique. Similarly, when 10uM were added to the cells, the cell viability reached 60 % for SFN-Emulsomes whereas a value of 75% was attained for the microsphere nano-formulation and was only able to reach 60 % only at a higher concentration of 25 uM.

On the other hand, in Soni et al., 2017, when Sulforaphane was loaded into a nano structured lipid carrier (NLC), the cell viability was observed to be 45% at 25 µM, which was approximately equal to the outcomes we got for the same concentration of SFN-Emulsomes (~47%). As for the 50 and 100 µM, the percentages were reported to be 25 and 20%, respectively. Similarly, values of 35% and 30% were observed for the same concentrations of the Emulsome nano-formulation. The similarities between the two outcomes goes back to the nano-formulations common properties nanostructured lipid carriers and emulsomes share. In which, SLNs are colloidal particles prepared from solid lipids (solid at room temperature and body temperature), surfactants, active ingredient and water. The SLN are defined as a crystalline solid lipid core matrix with a mean diameter of 50-1000 nm, which is stabilized by surfactants (emulsifiers). Therefore, it differs from emulsomes in the outermost shell composition. Nonetheless, when formulated with PL and triglyceride (TG), SLNs have exactly the same composition as emulsomes [100]. Thereby, the resemblances in both compositions and cell viability results further endorse the authenticity of our outcomes.

Table 4.5: Cell Viability percentages for various concentration of different nano-formulations of SFN taken from the literature for comparison purposes.

Samples of SFN against MCF-7 cell line	Time of incubation	Concentration (μM)	Cell Viability (%)
SFN–PLGA microsphere	24 hours	5 μM	98 %
		10 μM	75 %
		20 μM	60 %
Sulforaphane Liposomes	72 hours	5.28 μM	82 %
		10.56 μM	70 %
(SFN)-loaded nano structured lipid carriers (NLC)	24 hours	25 μM	45 %
		50 μM	25 %
		100 μM	20 %
SFN with PEGylated gold coated Fe3O4 magnetic nanoparticle	72 hours	6 μM	55 %
		9 μM	40 %
		15 μM	20 %
Sulforaphane mPEG–PCL co- polymer nanoparticles	24 hours	18 μM	75 %
	48 hours	18 μM	50 %
	72 hours	18 μM	37 %
Sulforaphane (SFN) loaded into gold nanorod mesoporous silica nanoparticles core- shell	48 hours	1.5 μM	75 %
		6 μM	65%
		24 μM	50 %
		48 μM	30 %

To further investigate our results, for Sulforaphane mPEG–PCL co-polymer nano-carrier, a cell viability percentage of 75 was attained for a concentration of 18uM, which means

the nanoparticle exhibited a weaker effect than ours, in which at 10uM and 25 uM dosages, the viability fluctuated between 55 and 60 % for SFN-Emulsomes.

Taking into consideration the cell viability analysis after 48 hours (**Figure 4.6**), mPEG–PCL co-polymer nano-carrier's outcomes and ours were closer in comparison, in which the 18 µM reached 50%, whereas SFN-Emulsomes fluctuated between (58% - 50%) for the corresponding concentrations of 10 and 25 µM. Which is also convenient for SFN loaded into gold nanorod mesoporous silica nanoparticles core-shell in Manjili et al., 2018, in which the cell viability decreased to 50% for the 24 µM concentration and 30% for the 48 µM concentration, analogous to SFN-emulsomes which reach 34% at 50 24 µM. In conclusion, the outcomes of SFN-emulsomes matched the ones found in the literature for several nano-formulation of the compound Sulforaphane, in which they all aided in highly decreasing the cell viability's percentage, reaching a minimal approximate value of 30% after the passage of 48 hours which confirms the anti-tumor properties of the Sulforaphane composite.

In addition to that, the fact that the SFN-Emulsomes were more effective after the passage of time is reliable when it is taken into consideration the controlled release of SFN from within the Emulsome or any other vehicle for that matter.

By following the MTT assay analysis concerning blank Emulsome, an undesirable decrease in cell viability was observed. This feature has never been detected in the literature before when blank emulsomes were tested on liver, pancreatic, and thyroid cancer [88]. To further investigate this effect, the nano-formulation were tested on the control group to observe its minimal side effect, which weighs nothing against the chemo-agents used clinically nowadays.

For example, when micelles (mPEG–PCL co- polymer) where tested on these cells, they caused a slight cell viability reduction reaching ~ 86 %, a similar 85 % value was observed when PCL-PEG-PCL micelles were utilized [101]. Also, an 80% cell viability was observed when gold nanoparticles were used[102]. In addition to that, Liposomes carriers exhibited a similar value falling within the range of 89% [103]. All these values certified the outcomes obtained in this study, in which the nano formula is bound to exhibit a little impact over the cells, but a one much less effective when compared to the drug encapsulated nano formulation. Which demonstrates that the nano-size range and

improved drug encapsulation with regular and comprehensive drug discharge, inquisitive into a probable enrichment for the antitumor properties of the compound.

Table 4.6: Cell Viability percentages for various concentration of different nano-formulations taken from the literature for comparison purposes.

Samples of nanoparticles against MCF-7 cell line	Cell Viability (%)
mPEG–PCL co- polymer nanoparticles	~ 86 %
Gold nanorod mesoporous silica nanoparticles core-shell	~ 80 %
PCL–PEG–PCL copolymeric-based micelles	~ 85 %
Liposomes nanocarriers	~ 89 %

To establish whether the SFN-nanoparticle exhibited a noticeable damage to the proliferation aptitude and cell sustainability of human breast cancer cells was correlated with the stimulation of apoptosis, cells were treated with free SFN, SFN-Emulsomes and Blank emulsomes. The apoptotic level was estimated through flow cytometry (**Figure 4.17**). The data obtained were compared with the findings in the literature. A 25 % of early apoptosis was observed for Sulforaphane-Emulsomes, which matched the outcomes when SFN was encapsulated within PEGylated Iron Oxide-Gold Nanoparticles [85]. It is important to shed the light on how the emulsome encapsulation amplified the effect of Sulforaphane on apoptosis. In which it increased from a 3% induction into 25 % for Free SFN and SFN-Emulsomes, respectively. Whereas Blank Emulsomes demonstrated no to faint percentage encompassing a value reaching 3 %. The IC50 found for free SFN in our studied varied from that in the literature. Although in the literature, each analysis exhibited values that are vastly different from one another. In which in Tracy et al., 2007, the IC50 value was 9.4 μM [104]which is 9 folds to what we obtained. However, in Tseng et al., 2004, the IC50 of SFN reached 40.5 μM which is also much higher than that of Tracy's and our outcome. Thereby, this difference doesn't disqualify the value obtained in our study.

CHAPTER 6

5. CONCLUSIONS AND FUTURE WORK

Earlier and continuing revisions have reported the big potential of Sulforaphane and its strong effect against cancer, but up till now, there isn't any research that took into consideration emulsome as carrier for this specific compound. Thus, the objective of this study was to encapsulate Sulforaphane into a lipoidal vesicular system comprising Sulforaphane inside a solid fat core surrounded by lipid multilayers, known as emulsome.

Overall, the MCF-7 cell lines tested showed a significant decrease in cell viability over the passage of 72 hours when Sulforaphane-emulsome was administrated. There was a slight decrease in the cell viability when blank emulsome was added, that's why further experimental studies were required to investigate the effect of blank emulsome on MCF-10a healthy cells, which showed a slight to no negative effect. Therefore, the profile established for this formulation is safe and it will need a few improvements regimens to be fit for in vivo model's studies and further on human trials that could investigate the profits of such treatment and define it as an efficient protective therapy against breast cancer.

As to expand the study, Western blot studies can be carried out to further investigate the genetic pathways Sulforaphane operates on in order to understand its interference in the cell cycle phases. In addition to that, Sulforaphane-Emulsome could be tested on several cancer cell types and compared all together to better comprehend Sulforaphane's mechanism of action and how it differs from one cell to another.

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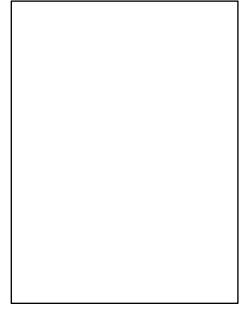
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DEVELOPMENT OF ANTI-CANCER NANOFORMULATIONS BY INCORPORATION OF WATER-INSOLUBLE NATURAL BIOACTIVE AGENTS INTO LIPID-BASED DRUG DELIVERY SYSTEMS